CHARACTERIZATION OF AN ANTISENSE OLIGONUCLEOTIDE TARGETED AT PHOSPHOLIPASE C GAMMA 1 IN THE MOUSE T-CELL LINE EL4.IL-2

CHOO HUI HWA

(B.Sc., NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2006

ACKNOWLEDGEMENTS

The research in this thesis would have taken far longer to complete without the help and encouragement from many others. It is a delight to acknowledge those who have supported me over the last three years.

First and foremost, I would like to express my deepest gratitude to my supervisor, Professor Fred Wong, for his patient guidance and assistance throughout the course of my master studies. Without his help and encouragement, I would not be able to overcome many unforeseen problems that occurred during this period and this work could never be possible.

I would also like to thank my co-supervisor, Professor Chang Chan Fong for his invaluable advice on my research work and for generously giving me access to his laboratory equipment.

I am grateful to Mr. Cheung Wai who taught me how to design a good set of primers. I am also very thankful to Mr. Tom Condon and Miss Lu Tao from Isis Pharmaceuticals for kindly sharing their experiences and transfection protocol.

Thanks to Professor Shazib Perviaz for allowing me to use the fluorescence polarization plate reader in his laboratory and also to Miss Andrea Lisa Holme who has been abundantly helpful in teaching me how to use the equipment.

I would also like to express my sincere gratitude to National University of Singapore for providing me with the scholarship and all the staffs in the Department of Pharmacology for their help.

The days would have passed far more slowly without the support of my friends and labmates, whom I thank for putting up with my shortcomings and for providing me with such a rich source of assistance, encouragement, conversation and entertainment.

Finally, I would like to express my deepest thanks to my parents and my husband for their endless love, support and encouragement, without whom I would not have been able to overcome all the challenges in my life.

Choo Hui Hwa

June 2006

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SUMMARY

Phospholipase Cγ1 (PLCγ1) plays an important role in T cell activation. Interaction of T cell with antigen results in activation of multiple protein kinases, which are in turn, linked to PLCγ1 activation and ultimately led to interleukin-2 (IL-2) production and T cell activation. Thus, PLCγ1 serves as an attractive target for molecular intervention. Therefore, in this present study, we identified and characterized an antisense oligonucleotide (ASO) to PLCγ1 as an attempt to interrupt T cell activation.

ASOs complementary to mouse PLCγ1 mRNA were designed and screened in the mouse T cell line, EL4.IL-2. Western blot analysis of transfected cell lysates showed that ASO3 demonstrated the highest antisense activity among other ASOs whereas the scrambled control oligonucleotide was without effect. In addition, ASO3 significantly reduced PLCγ1 mRNA in a concentration-dependent manner without altering PLCγ2 expression. Time course study on ASO3-mediated PLCγ1 gene inhibition profile revealed maximum mRNA downregulation at 6 h after electroporation and maximum protein reduction at 24 h posttransfection. Subsequent measurement of IP_3, intracellular calcium, and IL-2 levels, in PLCγ1 ASO-treated murine EL4.IL-2 cells, showed significant reduction in all the three parameters. Further study showed that pretreatment of murine EL4.IL-2 cells with PLCγ1 ASO, before anti-CD3/CD28 stimulation, significantly reduced EL4.IL-2 proliferation. However, unexpectedly, scrambled control oligonucleotide-treated cells also exhibited reduced proliferation. Therefore, further investigations involving the use of oligonucleotides associated with less nonspecific effects such as chimeric oligonucleotides, and additional controls such as sense and mismatch controls are needed to confirm the anti-proliferative effect of ASO3.

Our present work discovered and characterized an ASO capable of downregulating PLCγ1 expression, and subsequently inhibiting TCR/CD28 stimulation-induced IL-2 production in the mouse T cell line, EL4.IL-2. More extensive gene-walking in future studies may identify an even more potent PLCγ1 ASO, capable of inhibiting mouse PLCγ1 expression and T cell activation to a greater extent. Future studies employing multiple ASOs or small interfering RNAs (siRNAs) targeting specifically at key molecules involved in T cell activation ─ PLCγ1 and perhaps PI3K, may discover promising therapeutics that could completely attenuate T cell activation. Taken together, our present findings implicate that antisense inhibition of PLCγ1 may have therapeutic potential for the treatment of T cell-dependent disorders.

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LIST OF ABBREVIATIONS

2′-MOE	2′-O-methoxyethyl
AP	alkaline phosphatase
APS	ammonium persulfate
ASO	antisense oligonucleotide
BCA	bicinchoninic acid
Bcl-2	B cell leukemia/lymphoma 2 (Bcl-2)
BSA	bovine serum albumin
cDNA	complementary DNA
CRAC	calcium release-activated Ca^{2+}
CsA	cyclosporine
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FDA	Food and Drug Administration
FK506	tacrolimus
Fura-2/AM	fura-2/acetoxymethylester
Grb2	growth factor receptor-bound protein 2
GRP	guanyl nucleotide-releasing protein
GVHD	graft-versus-host disease
HBSS	Hank's balanced salt solution
HIV-1	human immunodeficiency virus type I
HRP	horseradish peroxidase

ICAM-1	intercellular adhesion molecule-1
IE2	immediate early region 2
Ig	immunoglobulin
IL-2	interleukin-2
IL-2R	IL-2 receptor
IP_3	Inositol 1,4,5-trisphosphate
IRS	insulin receptor substrates
ITAMs	immunoreceptor tyrosine-based activation motifs
Itk	inducible T cells kinase
JAKs	Janus family of protein tyrosine kinases
LAT	linker for activation of T cells
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NFκB	nuclear factor κB
NFAT	nuclear factor of activated T cells
NPs	N3´-P5´ phosphoramidates
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCA	perchloric acid
PES	phenazine ethosulfate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C

PLCγ1	phospholipase C gamma 1
PMSF	phenylmethylsulfonyl fluoride
PNAs	peptide nucleic acids
PS	phosphorothioate
PTB	phosphotyrosine-binding
PTK	protein tyrosine kinase
PVDF	polyvinylidene fluoride
RT-PCR	reverse transcription-polymerase chain reaction
Scr	scrambled control oligonucleotide
SDS	sodium dodecyl sulphate
SH2	Src homology domain 2
SLP-76	SH2-containing leukocyte protein
SOS	son of sevenless
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzidine
TTBS	Tween 20-Tris-buffered saline
USFDA	United States Food and Drug Administration

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

Publications

1. **Choo HH**, Wong WSF. Characterization of an antisense oligonucleotide targeted at phospholipase C gamma 1 in the mouse T-cell line EL4.IL-2. Currently writing.
2. Duan W, Chan HP, McKay K, Crosby JR, **Choo HH**, Leung BP, Karras JG, Wong WSF. Inhaled p38α mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice. Am J Respir Crit Care Med 2005;171:571-578.
3. Li H, Cheung W, **Choo HH**, Chan HP, Lai PS, Wong WSF. IL-10 synergistically enhances GM-CSF-induced CCR1 expression in myelomonocytic cells. Biochem Biophys Res Commun 2003;304:417-424.
4. Tsang F, **Choo HH**, Dawe GS, Wong WSF. Inhibitors of the tyrosine kinase signaling cascade attenuated thrombin-induced guinea pig airway smooth muscle cell proliferation. Biochem Biophys Res Commun 2002;293:72-78.

Conference Abstract

1. **Choo HH**, Chan HP, Wong WSF. PLCγ1 antisense oligonucleotide blocks T cell activation in murine EL4.IL-2 cells. Combined Scientific Meeting 2005 Singapore, S224, 2005.

1. INTRODUCTION

1.1. T cell activation

T cells respond to antigen stimulation by producing cytokines, including interleukin-2 (IL-2) and undergoing clonal expansion (Kovalev et al., 2001). Efficient activation of T cells requires the engagement of both the T cell receptor (TCR) complex and the CD28 costimulatory receptor with their appropriate ligands (Lenschow et al., 1996; Chambers and Allison, 1999). Stimulation of the TCR complex alone is insufficient to activate T cells completely, rather causing a state of clonal anergy (Ullman et al., 1990; Crabtree and Clipstone, 1994). Additional signals from CD28 costimulation, which enhance the TCR-mediated signals, are necessary for T cells to become optimally activated (Powell et al., 1998) (Figure 1).

1.1.1. TCR-CD28 signaling in T lymphocytes

1.1.1.1.TCR-mediated signal transduction

The TCR complex of transmembrane proteins includes the TCR, a heterodimer of alpha/beta subunits, that recognizes antigen, and a set of proteins called CD3 involved in signal transduction (Figure 2). CD3 associated with the T cell receptor includes six polypeptides arranged in three dimers. The cytoplasmic domains of these polypeptides have in common a sequence motif known as ITAMs (immunoreceptor tyrosine-based activation motifs), that form the structural basis for interactions with downstream molecules, and play a critical role in transducing extracellular signals from TCR to downstream signaling molecules (Reth et al., 1989; Wagner et al., 1992). The earliest events in TCR signaling are dependent on tyrosine kinases of the Src and Syk families and eventually leading to the activation of the phospholipase C gamma 1 (PLCγ1) pathway and Ras pathway, events crucial for activation of transcription factors regulating IL-2 gene expression (Baker et al., 2001) (Figure 3).

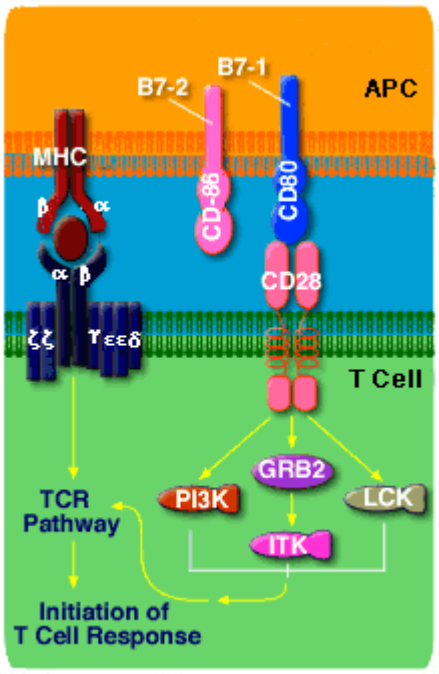

Figure 1. TCR-CD28 costimulation during T cell activation (adapted from www.biocarta.com/)

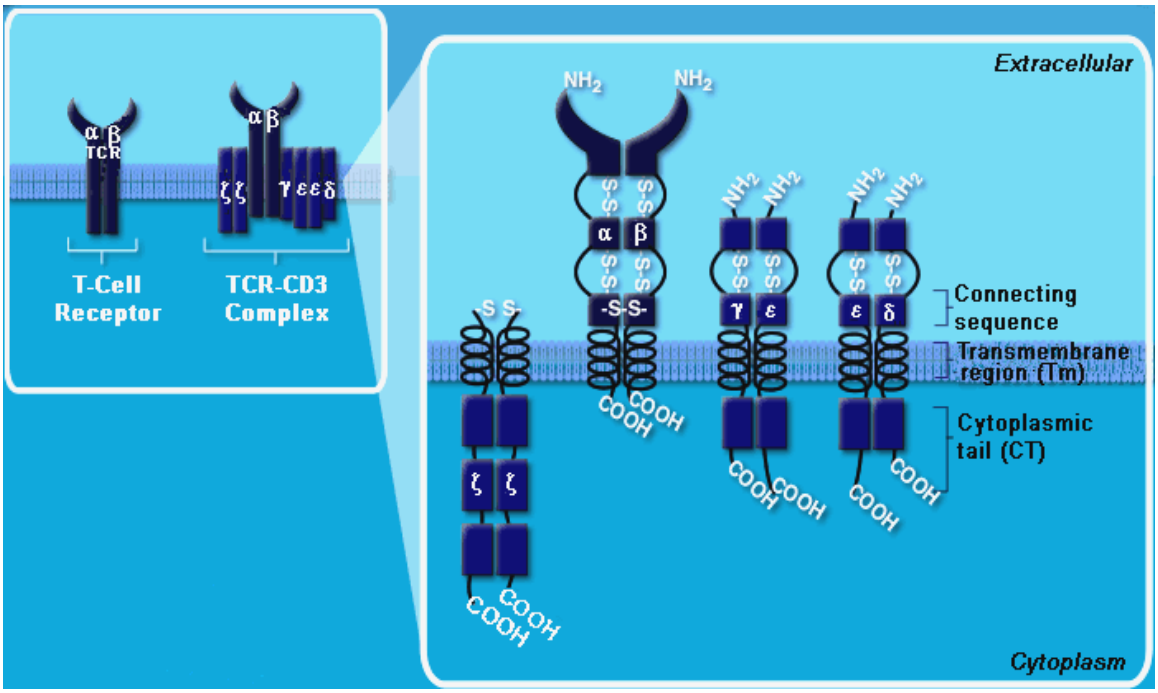

Figure 2. The TCR complex (adapted from www.biocarta.com/)

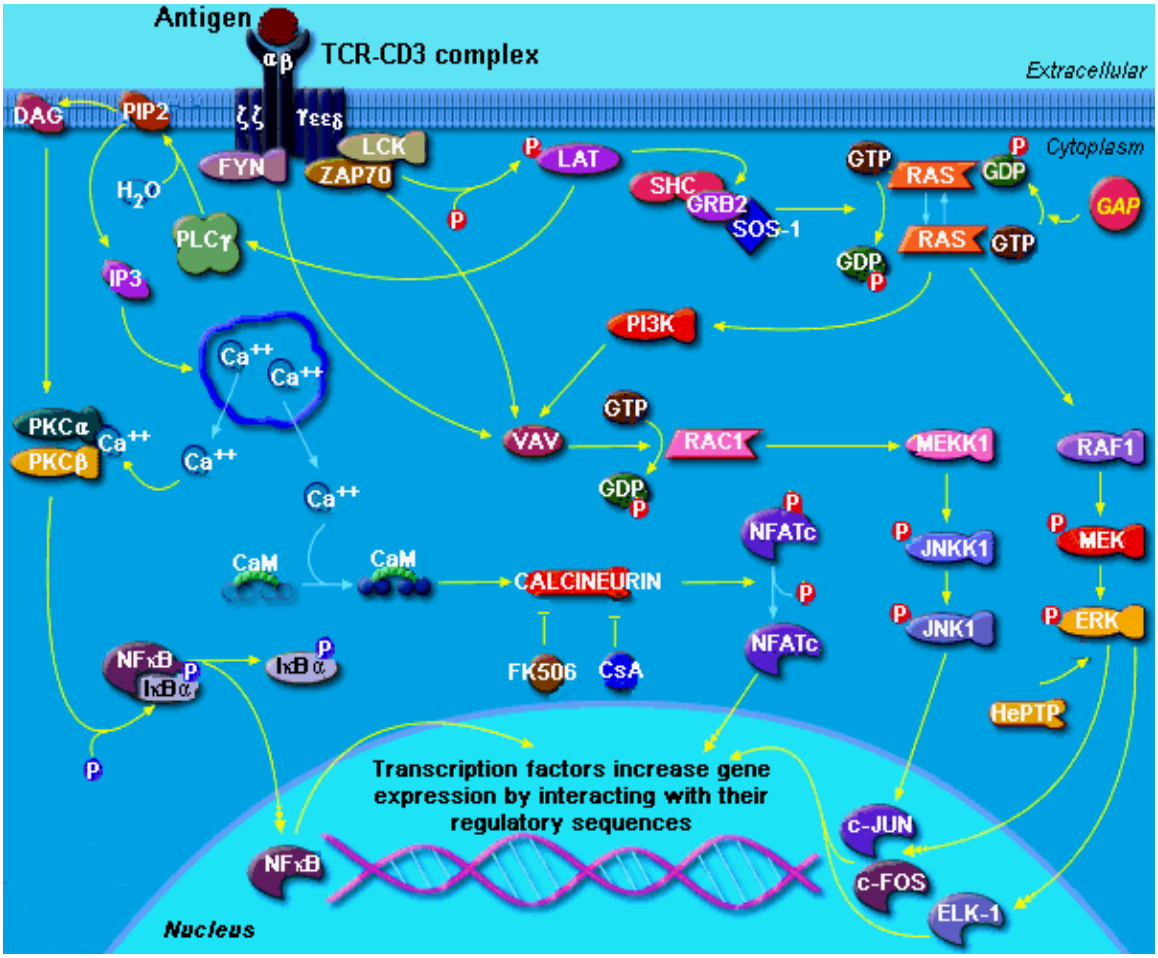

Figure 3. T cell receptor signaling pathway (adapted from www.biocarta.com/)

Ligation of the TCR stimulates the activation of two Src-family of nonreceptor protein tyrosine kinases (PTKs): Lck and Fyn. Activated Lck and Fyn phosphorylate tyrosine residues contained within ITAMs of the CD3 chains. The phosphorylated residues serve as docking sites for the tandem Src homology domain 2 (SH2) domains of the Syk family PTK, ZAP-70 (Chan et al., 1992). ZAP-70 is subsequently phosphorylated and activated by Lck. These two kinases phosphorylate 2 major adaptor molecules, linker for activation of T cells (LAT) and SH2-containing leukocyte protein (SLP-76) (Baker et al., 2001). This event results in the assembly of LAT with downstream signaling molecules such as PLCγ1, growth factor receptor-bound protein 2 (Grb2), and Gad-SLP-76 complex, and so forth (Gilliland et al., 1992; Buday et al., 1994; Sieh et al., 1994; Dewulf et al., 1995). This is thought to be required for PLCγ1 tyrosine phosphorylation and activation, as well as the activation of Ras. It has been proposed that the LAT-associated complex colocalizes PLCγ1 with the inducible T cells kinase (Itk), which in turn phosphorylates and activates PLCγ1 (Perez-Villar and Kanner, 1999; Bunnell et al., 2000; Myung et al., 2000; Wange et al., 2000; Zhang and Samelson, 2000).

Activated PLCγ1 cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Weiss et al., 1991). The former product regulates the levels of intracellular Ca^{2+}, whereas the latter activates protein kinase C (PKC), and Ras-guanyl nucleotide-releasing protein (GRP) that activates Ras (Kazanietz et al., 2000). IP_3 binds to IP_3 receptors on the endoplasmic reticulum, leading to the release of Ca^{2+} into the cytoplasm, this in turn triggers entry of extracellular Ca^{2+} through calcium release-activated Ca^{2+} (CRAC) channel (Sasaki

and Hasegawa-Sasaki, 1985; Abraham et al., 1988; June et al., 1990; Scharenberg and Kinet, 1998). The rise in cytoplasmic Ca^{2+} leads to the activation of calmodulin and calcineurin (serine/threonine phosphatase). Activated calcineurin then dephosphorylate cytoplasmic nuclear factor of activated T cells (NFAT), allowing it to translocate into the nucleus and act together with other nuclear transcription factors to activate IL-2 expression (Crabtree et al., 1989; Clipstone and Crabtree, 1992; Jain et al., 1993; Woodrow et al., 1993; Crabtree and Clipstone, 1994).

DAG is an activator of Ras-GRP and several of the members of the serine/threonine PKC family. Ras activation by Ras-GRP results in the sequential activation of Raf-1, mitogen-activated protein kinase kinase (MEK) and mitogen-activated protein kinase (MAPK), which eventually leads to the activation of nuclear transcription factors, Fos and Jun (AP-1) (Pulverer et al., 1991; Marais et al., 1993). Activation of PKC leads to the activation of nuclear factor κB (NFκB) and Raf-1 that contributes to AP-1 activation via activation of the MAPK pathway (Kolch et al., 1993; Carroll and May, 1994; Ueda Y et al., 1996; Marais et al., 1998). In addition to activation of MAPK pathway via DAG, another mechanism involves Ras activation via the recruitment of Grb2-associated son of sevenless (SOS), a guanine nucleotide exchange factor for Ras, to the plasma membrane by virtue of the ability of the SH2 domains of Grb2 to bind to membrane-resident, tyrosine-phosphorylated proteins such as LAT (Clements et al., 1999; Myung et al., 2000; Wange et al., 2000; Zhang and Samelson, 2000).

Hence, signaling through TCR leads to the eventual activation of transcription factors, NFκB, AP-1 and NFAT, which work together to regulate IL-2 gene expression.

1.1.1.2.CD28 costimulation

Additional signals delivered by the major costimulatory molecule, CD28 are necessary to enhance the TCR-mediated signals to completely activate T cells, resulting in IL-2 secretion, cellular proliferation and prevention of anergy (June et al., 1994). The CD28-specific signaling pathway is not well defined. Several studies on CD28 costimulation suggest that CD28 probably activates the phosphatidylinositol 3 kinase (PI3K) and Akt kinase pathway (Parry et al., 1997; Kane et al., 2001), Lck, growth factor receptor-bound protein 2 (Grb2)/inducible T cells kinase (Itk), the Rho family GTPase Rac, NFκB (Rudd et al., 1996), c-Jun N-terminal kinase (JNK) (Su et al., 1994), and PLCγ1 pathway (Wells et al., 2003), to provide its costimulatory signal for T cell activation. CD28 costimulation enhances TCR-mediated signals in IL-2 expression by enhancing the stability of the IL-2 mRNA as well as IL-2 transcription. The signal provided via CD28 is also necessary for induction of clonal expansion and prevention of T cell anergy as CD28 blockade suppresses T cell proliferation, induces unresponsiveness and cell death (Powell et al., 1998).

1.1.2. IL-2 gene regulation and signaling in T lymphocytes

Initiation of IL-2 gene transcription is a hallmark of T-cell activation. Consequently, IL-2 functions to stimulate clonal expansion of T cells and to promote T-cell-mediated immune responses (Jan and Kaminski 2001).

1.1.2.1.Regulation of IL-2 gene expression

Resting T cells exhibit almost no basal-level IL-2 expression. The IL-2 gene is tightly regulated at the transcriptional level by several *cis*-acting elements in the proximal promoter/enhancer region of the gene, which are recognized by AP-1, NFκB and

NFAT transcription factors, induced by TCR/CD28 costimulation. These transciption factor recognition sequences inside the IL-2 promoter are atypical sites that do not allow the stable binding of each transcription factor alone. The gene is fully expressed only if its promoter is fully occupied by all three inducible transcription factors, each of which stabilizes binding of the others (Rothenberg and Ward, 1996; Lindholm et al., 1999). The result is that TCR/CD28-mediated signaling cascades synergistically activate the proliferation of the cell (Hughes and Pober, 1996).

1.1.2.2.IL-2 signaling via the IL-2 receptor

Upon activation, T cells produce IL-2 and the secreted IL-2 binds to the IL-2 receptor (IL-2R) on the T cell surface, promoting proliferation in an autocrine manner (Rooney et al., 1995). The IL-2R is formed from three subunits: alpha (α), beta (β) and common gamma (γ_c) chain (Figure 4). The IL-2R lacks enzymatic activity, instead it coordinates with the Janus family of protein tyrosine kinases (JAKs). JAK3 preferentially binds the γ_c subunit while the IL-2Rβ subunit binds JAK1 (Russell et al, 1994).

Binding of IL-2 to the IL-2R brings the receptor subunits into proximity; this in turn activates the JAKs. Upon activation, the JAKs phosphorylate the receptor subunits, providing docking sites for proteins with SH2 or phosphotyrosine-binding (PTB) domains. The signal transducer and activator of transcription (STAT) family of transcription factors is a class proteins that contain SH2 domains. STAT3 and STAT5 bind to phosphorylated tyrosine residues on the receptor subunits and are in turn phosphorylated by JAKs. Next, they dimerize through phosphotyrosine-SH2 interactions, and translocate to the nucleus where they regulate gene expression (Schindler and Darnell, 1995; Johnston et al., 1996; Benczik and Gaffen, 2004).

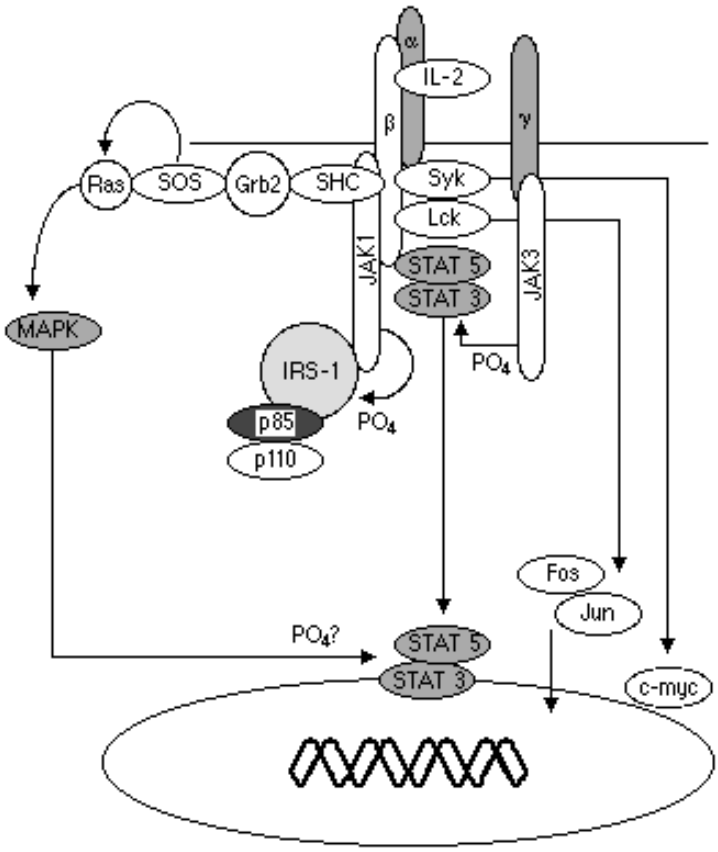

Figure 4. IL-2 signaling pathway (adapted from O'Shea et al., 2000)

Another important substrate that is tyrosine phosphorylated in response to IL-2 is the adaptor molecule, SHC. SHC binds to the phosphorylated receptor via its PTB domain and then recruit Grb2 and SOS. This in turn activates the Ras/Raf/MAPK pathway, leading to the phosphorylation and activation of cytoplasmic and nuclear substrates. MAPK phosphorylate STAT proteins on serine residues, enabling them to regulate gene expression (Pelicci et al., 1992; Ihle et al., 1995, Benczik and Gaffen, 2004).

IL-2 also induces phosphorylation of the insulin receptor substrates (IRS) 1 and 2, which provide numerous docking sites for proteins that bind phosphorylated tyrosine residues. IRS molecules may also serve to couple to the Ras/Raf pathway (Skolnik et al., 1993; Johnston et al., 1995). Other tyrosine kinases, such as members of the Src family (including Lck and Lyn) and Syk, are also activated by IL-2. They appear to be important in the activation of transcription factors such as Myc, Fos and Jun (Taniguchi, 1995).

1.1.3. Inhibitors of TCR-mediated signaling

Inhibition of TCR-mediated signaling is one of the working mechanisms of many immunosuppressive drugs (Kang et al., 2003). Immunosuppressive drugs have wide applications in the prevention of allograft rejection in organ transplants, in the prevention of graft-versus-host disease (GVHD) in bone marrow and stem cell transplants, and in therapy for chronic autoimmune inflammatory conditions (Bierer et al., 1993; Xu et al., 1995; Goldman et al., 2000). Immunosuppressive agents such as hydroxychloroquine, cyclosporin A (CsA), tacrolimus (FK506), and glucocorticoids were found to inhibit TCR-induced signaling events. Hydroxychloroquine inhibits TCR-induced intracellular calcium mobilization by

reducing the endoplasmic reticulum calcium store size (Goldman et al., 2000). CsA and FK506 bind to intracellular immunophilin proteins and inhibit calcineurin phosphatase activity, which results in the inhibition of nuclear translocation of NFAT and IL-2 production (Bierer et al., 1993). Glucocorticoids suppressed TCR-induced proliferation of T cells, through downregulation of c-Fos expression and inhibition of AP-1, NFAT and NFκB transcriptional activity (Barnes and Adcock, 1993; Paliogianni et al., 1993). These drugs although have wide applications, are associated with potentially significant side effects owing to systemic toxicity, and the drawback of resistant activated T cells (Goldman et al., 2000; Cristillo et al., 2003; Tsitoura and Rothman, 2004).

1.1.4. Role of PLCγ1 in T cell activation

PLCγ1 is the predominant isoform in T cells and is required for T cell function (Wang et al., 2000; Wilde and Watson, 2001). Cumulative evidence supports the fact that PLCγ1 plays a pivotal role in T cell activation (Figure 5) (Diaz-Flores et al., 2003). Studies using PLCγ1-deficient cell lines demonstrated failure to activate NFκB in response to T cell costimulation (Dienz et al., 2003), and impaired TCR activation (Irvin et al., 2000). In addition, Kang and colleagues (2003) reported that Rosmarinic acid, a hydroxylated compound found in herbal plants, which blocks Itk and PLCγ1 activation, inhibits IL-2 expression and subsequent T cell proliferation. Similarly, mutations in the T cell adapter LAT, which prevents PLCγ1 recruitment and activation (Finco et al., 1998), abrogate expression of IL-2 (Crabtree et al., 1989). The relevance of PLCγ1 in CD28 costimulation was highlighted by Wells and colleagues (2003), who demonstrated that PLCγ1 activation was impaired in cells anergized by costimulatory blockade, and complete T cell activation could be restored using

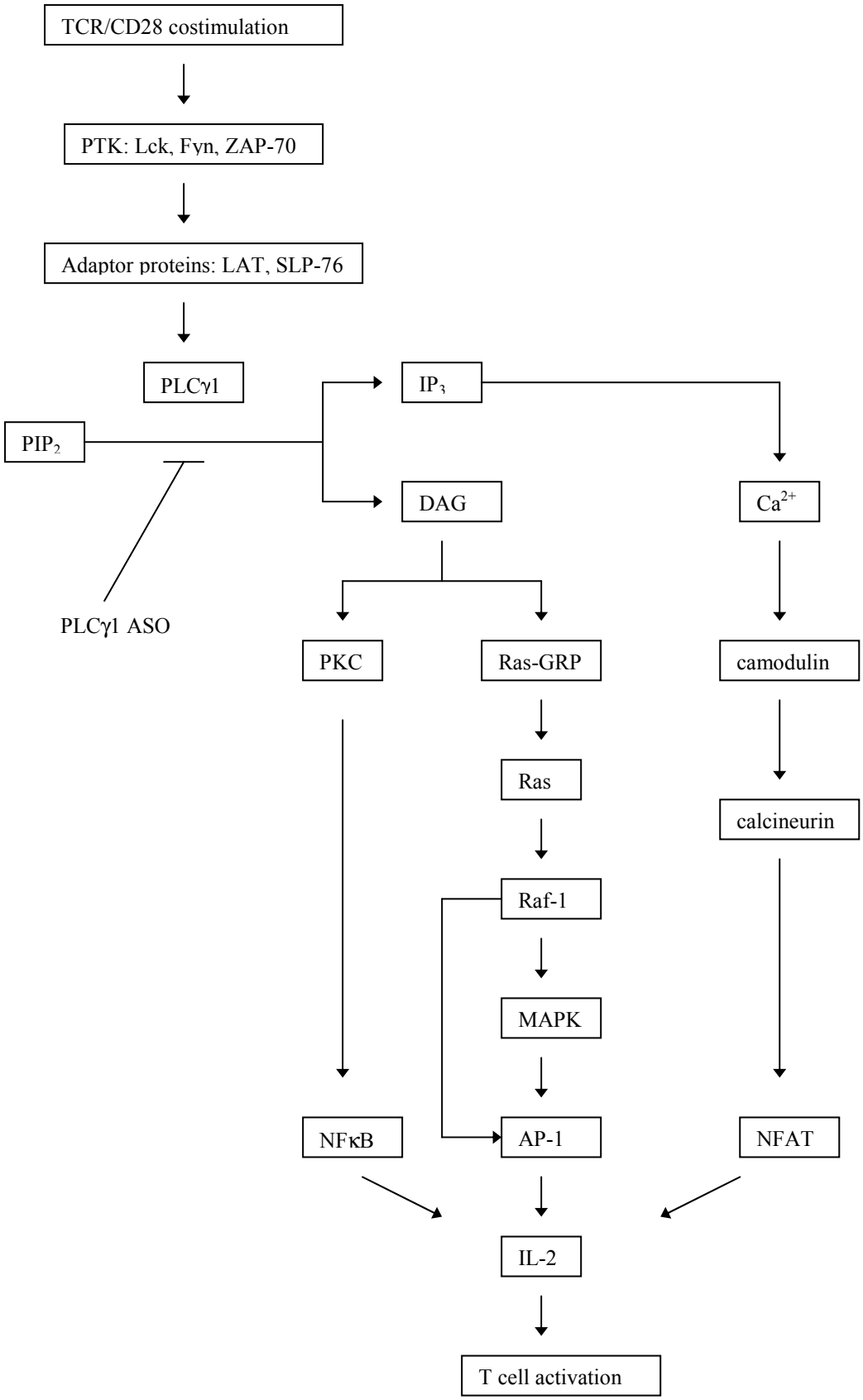

Figure 5. Schematic diagram illustrating the pivotal role PLCγ1 plays in T cell activation.

analogs that mimic actions of DAG and IP_3, the immediate products of PLCγ1 activity.

The prominent role PLCγ1 plays in T cell activation is mediated through the immediate products of its activity, IP_3 and DAG, and their respective downstream targets, Ca^{2+}, PKC and Ras-GRP (Kazanietz et al., 2000). The importance of Ca^{2+} and DAG in T cell activation was demonstrated by Truneh and colleagues (1985), who showed that a combination of Ca^{2+} ionophore and phorbol esters, which function as DAG analogues, could mimic TCR signals, leading to full T cell activation.

Hence, inhibition of PLCγ1 would be expected to attenuate T cell activation. It therefore represents an attractive target for antisense technology, with significant potential as an immunosuppressive agent, useful for the specific treatment of T cell-mediated autoimmune diseases and for the prevention of allograft rejection and GVHD, and therapy of chronic autoimmune inflammatory conditions in individuals who are non-responsive to conventional immunosuppressive therapy.

1.2. Antisense technology

1.2.1. Antisense oligonucleotides (ASOs) – an alternative to small molecule inhibitors

The use of ASOs as both experimental tools and therapeutic molecules has emerged as a powerful alternative to small molecule inhibitors employing traditional drug design strategies (Bennett and Cowsert, 1999; Cooper et al., 1999; Patil et al., 2005). To date, there is one Food and Drug Administration (FDA)-approved antisense drug, Vitravene™ (Isis Pharmaceuticals), already in the clinic for the treatment of

cytomegalovirus retinitis, and numerous other antisense drug candidates are in advanced stages of human clinical trials (Table 1). Affinitak and Alicaforsen (Isis Pharmaceuticals) are now in phase 3 clinical trials for non-small cell lung cancer and Crohn's disease respectively. Genasense, developed by Aventis and Genta is being investigated in advanced phase 3 trials, in combination with other chemotherapy regimens, for a range of cancers including malignant melanoma, chronic lymphocytic leukemia and multiple myeloma (Opalinska and Gewirtz, 2002; Patil et al., 2005; Vidal et al., 2005;).

ASOs are short stretches of synthetic, chemically modified, DNA designed to hybridize to specific mRNA sequences present in the gene that one wishes to inhibit. Specific interaction of ASO with the targeted mRNA by Watson-Crick base pairing leads to inhibition of translation of the protein encoded by the targeted transcript (Crooke and Bennett, 1996; Gewirtz et al., 1997; Crooke et al., 1998; Galderisi et al., 2001;) (Figure 6). This exploitation of nature's use of hydrogen bonding to allow mRNA to recognize complementary DNA imparts tremendous specificity of action to ASOs, which is not readily attainable with small molecule inhibitors (Cooper et al., 1999; Patil et al., 2005). In addition, unlike small molecule inhibitors, the antisense approach only requires knowledge of the nucleic acid sequence information of the target gene, and does not require knowledge of the function, structure, or localization of the protein of interest. Thus, ASOs can be developed and applied against any gene as long as the genetic information is available (Cooper et al., 1999; Roth and Yarmush, 1999). These characteristics make antisense oligonucleotides attractive as tools for target validation, and as therapeutics to selectively modulate the expression of genes involved in the pathogenesis of diseases (Aboul-Fadl et al., 2005).

Table 1. ASO therapeutics currently in advanced stages of clinical development (phase 3 and beyond) (adapted from Patil et al., 2005)

Drug Candidate - Company	Development Status*	Molecular Basis of Action	Disease Indication
Vitravene – Isis Pharmaceuticals	FDA approved	Inhibitor of immediate early region 2 (IE2) of human cytomegalovirus	Cytomegalovirus retinitis in AIDS patients
Affinitak - Isis Pharmaceuticals	Phase 3 in combination with carboplatin and paclitaxel	Inhibitor of PKC-alpha expression	Stage IIIb or Stage IV non-small cell lung cancer
Alicaforsen - Isis Pharmaceuticals	Phase 3	Inhibitor of intercellular adhesion molecule-1 (ICAM-1)	Crohn's disease
Genasense – Aventis and Genta	Late stage phase 3 in combination with dexamethasone	Inhibitor of B cell leukemia/lymphoma 2 (Bcl-2) protein	Malignant melanoma
Genasense – Aventis and Genta	Phase 3 in combination with fludarabine and cyclophosphamide	Inhibitor of Bcl-2 protein	Chronic lymphocytic leukemia
Genasense – Aventis and Genta	Phase 3 in combination with dacarbazine	Inhibitor of Bcl-2 protein	Multiple myeloma

*All development statuses are filed with the United States Food and Drug Administration (USFDA).

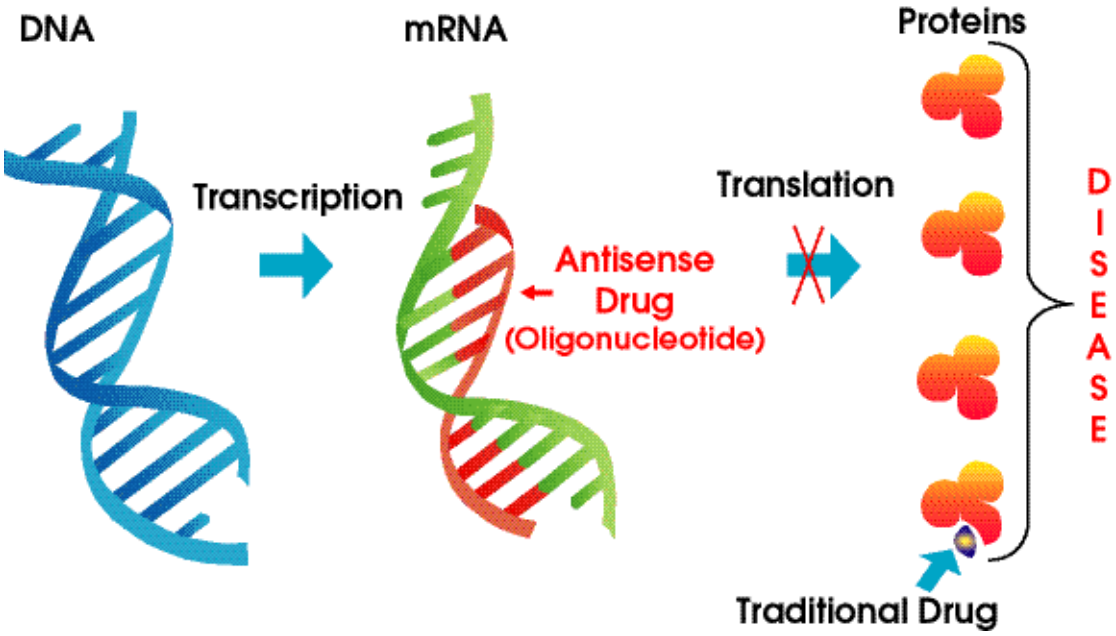

Figure 6. Principle of antisense technology (adapted from http://cmbi.bjmu.edu.cn/)

1.2.2. Molecular mechanisms of ASOs

ASO interacts and forms a duplex with target mRNA and inhibits its corresponding protein biosynthesis. There are several mechanisms that can result in inhibition of target protein expression. Mechanisms that are triggered as a result of duplex formation are dependent on the nature of the antisense molecules used for mRNA targeting (Gewirtz et al., 1998; Cooper et al., 1999).

ASOs of many, but not all, types (see next section on chemical modifications of ASOs) support the binding of RNase H at sites of RNA-DNA duplex formation. Such binding is an important effector of antisense actions because once bound, RNase H, a ubiquitous enzyme functions as an endonuclease that recognizes and cleaves the RNA in the RNA-DNA duplex resulting in degradation of the cleaved mRNA by exonucleases. Following the destruction of target mRNA, translation of the corresponding protein cannot occur. Of significant interest is the fact that the ASO in the duplex is undamaged by the action of RNase H. Therefore, it is free to hybridize with multiple mRNA molecules, leading to their destruction in a catalytic manner (Gewirtz et al., 1998; Cooper et al., 1999; Vickers et al., 2003) (Figure 7).

Although activation of RNase H is the principal mechanism of action of many ASOs, some types of ASOs (see next section) do not support the activity of this enzyme at all. These ASOs inhibit target protein expression by means of steric hindrance. For example, they bind to the translation initiation codon or 5'-untranslated region on the target mRNA and block ribsome assembly, resulting in translational arrest. Alternatively, they are designed to interact with splicing sequences on pre-mRNA and inhibit its processing and transport from the nucleus, consequently inhibiting target

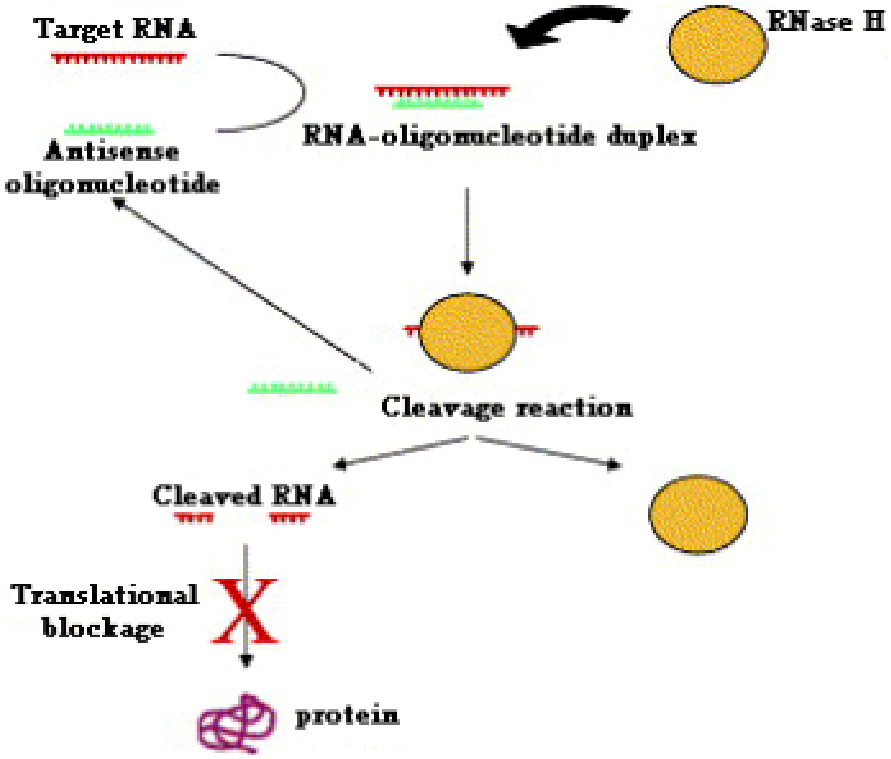

Figure 7. Prinicpal mechanism of action of most ASOs, involving activation of RNase H, which cleaves the targeted RNA and releases the ASO (adapted from Patil et al., 2005)

protein synthesis (Tidd et al., 1998; Crooke et al., 1999; Crooke et al., 2001). It is generally accepted that ASOs that are unable to induce RNase H activity are less effective inhibitors of translation than RNase H-active ASOs that induce irreversible destruction of the message, unless they form highly stable DNA/RNA complex (Bonham et al., 1995; Faira et al., 2001).

1.2.3. Chemical modifications of ASOs

Oligonucleotides having the endogenous phosphodiester backbone are susceptible to degradation by nucleases and hence have limited use for antisense applications (Engels and Uhlmann, 2000). A wide range of oligonucleotide chemical modifications to the nucleoside, the sugar, and the phosphate backbone has been explored to improve oligonucleotide stability. This has led to the development of the first and subsequently, second generation ASOs that have successively improved on the properties of previous ASOs (Vidal et al., 2005).

The most attention has been focused on two classes of analogues: phosphorothioate backbone-modified oligonucleotides and oligonucleotides modified at the 2′-O position of the sugar ring (Figure 8). Most prevalent among the oligonucleotide analogues are phosphorothioate (PS) oligonucleotides, in which a single oxygen atom in the phosphodiester bond is replaced by a sulfur atom (Roth and Yarmush, 1999; Vidal et al., 2005). These first generation ASOs are very widely used because of several desirable properties. First, PS ASOs exhibit ~100- to ~300-fold increase in nuclease resistance compared with phosphodiester oligonucleotides (Cooper et al., 1999; Crooke et al., 1999; Roth and Yarmush, 1999). In addition, PS ASOs have significantly increased biological half-life compared to their corresponding unmodified phosphodiester oligonucleotides (Patil et al., 2005). It is also noteworthy

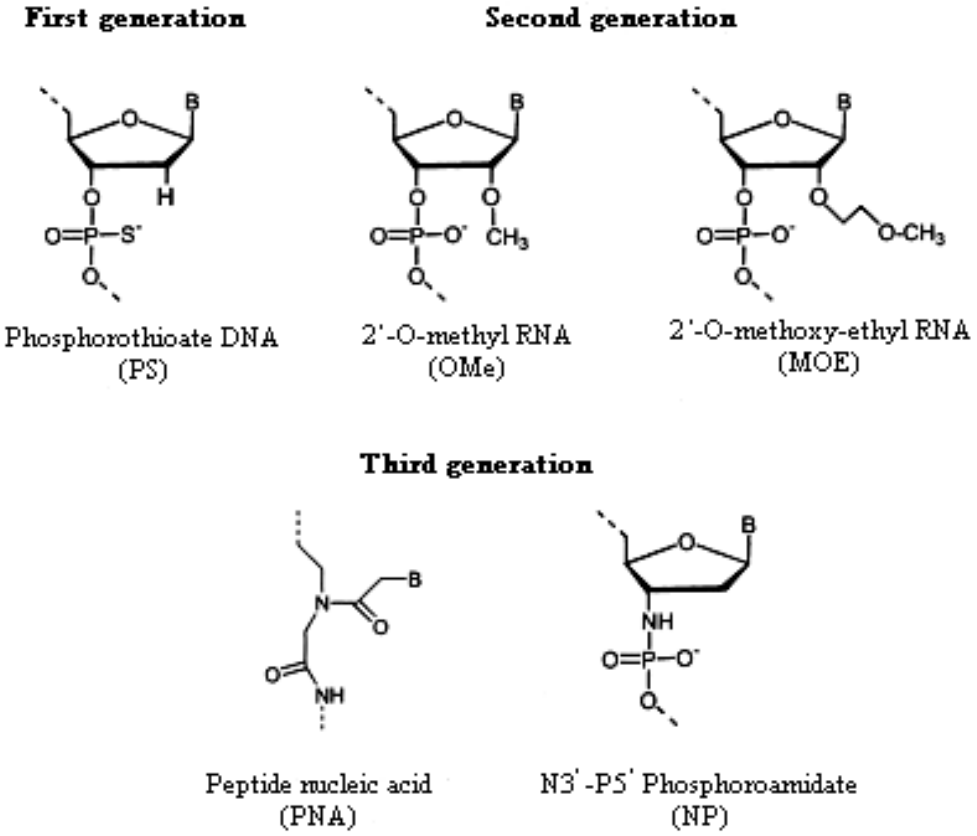

Figure 8. Types of chemical modifications for ASOs (adapted from Kurreck et al., 2003)

that PS ASOs support RNase H activity in the duplex, which plays an important role in irreversible destruction of target mRNA (Gewirtz et al., 1998). Nevertheless, PS ASOs suffer the drawbacks of reduced affinity for their complementary mRNA target in comparison to phosphodiester oligonucleotides (Cooper et al., 1999; Roth and Yarmush, 1999) and greater binding to proteins, leading in many cases to nonspecific effects (Stein et al., 1996).

Changes in the ASO sugar moiety can increase affinity for target mRNA and confer nuclease resistance. These modifications have been utilized in the generation of second generation ASOs. The most promising modifications are the insertion in position 2 of either 2′-O-methyl, 2′-O-methoxyethyl (2′-MOE), or 2′-O-alkyl group (Gewirtz et al., 1998; Vidal et al., 2005). Nevertheless, these 2′-O-modified ASOs do not support RNase H activity and, as a result, are less potent inhibitors of gene expression than the corresponding unmodified or PS-modified species (Gertwirtz et al., 1998; Roth and Yarmush, 1999). This has led to the use of chimeric oligonucleotides, in which a combination of 2′-O-modified and unmodified bases (or PS-modified bases) is used to impart affinity, resistance to nucleases, and activation of RNase H in one molecule. The minimum length required for RNase H activation has been found to be five bases. In this strategy, 2′-O-modified bases are used on the 5′- and 3′-ends of the oligonucleotide, which are most critical to nuclease resistance and initiation of hybridization, and a stretch of five to seven unmodified or PS-modified bases is used in the middle of the molecule to activate RNase H (Monia et al., 1993, 1996; Hill et al., 1999) (Figure 9).

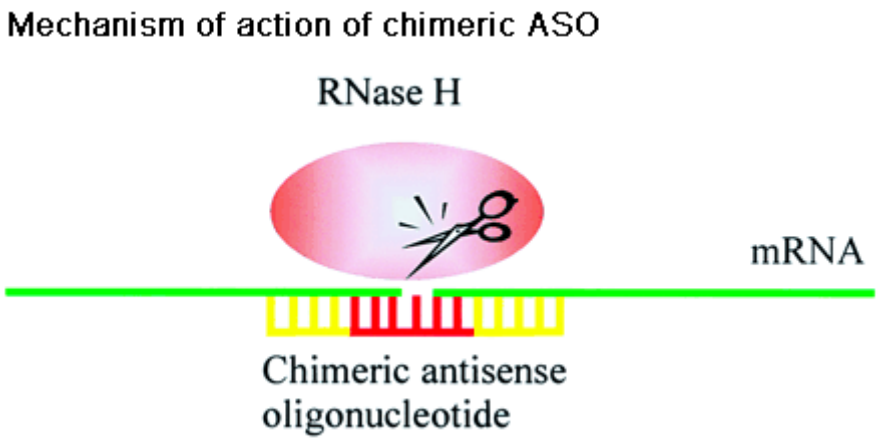

Figure 9. RNase H cleavage of target mRNA induced by chimeric ASO (adapted from Kurreck et al., 2003)

Many other chemical modifications are currently being evaluated. Two of the more interesting modified ASOs in the third generation are the N3′-P5′ phosphoramidates (NPs) and the peptide nucleic acids (PNAs) (Figure 8). The phosphoramidate modification is another example of oligonucleotide phosphate backbone modification, in which the 3′-hydroxyl group of the deoxyribose ring is replaced by a 3′-amino group (Chen et al., 1995). This modification creates a highly nuclease-resistant ASO with a high affinity towards the target mRNA (Gryaznov and Chen, 1994). Although the ability of the NPs to activate RNase H is weak in comparison to unmodified oligonucleotides (DeDionisio and Gryaznov, 1995), they effectively inhibit translation by steric blocking due to the highly stable DNA/RNA hybrids formed (Faira et al., 2001).

In PNAs, the sugar-phosphate backbone is completely replaced with a peptide-based backbone (Nielsen et al., 1993). Such compounds are completely nuclease resistant as they have no phosphodiester linkages. However, PNAs do not activate RNase H, they exert their antisense effects by steric blocking which, as in the case of 2′-O-modified ASOs, may not be as efficient as destruction of the mRNA (Gewirtz et al., 1998).

1.2.4. ASOs as research tools

1.2.4.1.Design of ASOs

The mechanism of action for ASOs requires that the oligonucleotide hybridize to its mRNA target. Therefore, in principle, design of ASO requires simply that the oligonucleotide be complementary to the mRNA. In practice, however, it has been found that not all oligonucleotide sequences are capable of reducing expression of their target mRNA, only a small percentage are effective inhibitors of gene expression

(Bennett et al., 1994; Dean et al., 1994; Duff et al., 1995; Alahari et al., 1996; Dean et al., 1996; Hill et al., 1999). Such observation may be explained by the highly complex folding that mRNA molecules undertake which render certain sequences inaccessible to oligonucleotides. In addition, RNA-binding proteins may also shield certain target mRNA sites (Cooper et al., 1999; Smith et al., 2000). Therefore, identifying effective antisense sequences is important for a successful antisense approach. It is possible to synthesize numerous complementary sequences and test for their antisense activity in a cell culture assay. However, this is not efficient because chance alone appears to dictate success, and is potentially expensive if many sequences have to be tested before a useful one is found (Bacon and Wickstrom, 1991; Bennett and Cowsert, 1999; Roth et al., 1999;).

A number of approaches have been developed to identify probable effective antisense sequences prior to cell-based screening to improve the "hit rates" and thus, reduce cost and time spent on antisense discovery (Cooper et al., 1999; Freier et al., 2001). Cell-free approaches have been used to identify optimal antisense sites in target mRNA. Typically, a library of randomized oligonucleotides complementary to the transcript is incubated with the target mRNA and RNase H. Mapping of the most favoured RNase H cleavage sites results in identification of the most favoured binding sites (Ho et al., 1996, 1998, 2000; Lima et al., 1997). DNA arrays have also been used to map target mRNA for hybridization-accessible sites (Southern et al., 1994; Milner et al., 1997). Although these methods can identify optimal binding sites in target mRNA and thus, probable effective antisense sequences, they are quite elaborate, and thorough screening of all possible oligonucleotides remain beyond current capabilities. These cell-free assays have been criticized on the grounds that the

structure of in vitro transcribed RNA molecules may be different from the actual mRNA structure inside a cell. Also, unlike in a cell-free assay, mRNA in the cell is bound to proteins, which render certain mRNA target sites unavailable to oligonucleotides (Cooper et al., 1999; Roth et al., 1999; Freier et al., 2001).

Theoretical strategies have also been employed to aid in the selection of effective ASOs (Stull et al., 1992; Sczakiel et al., 1993; Smith et al., 2000). Software have been developed to calculate and predict mRNA secondary structure, single-stranded regions in target mRNA secondary structure and hybridization thermodynamics. These provide useful information on hybridization-accessible sites on target mRNA and affinity of oligonucleotide to target mRNA and thus have been used to identify probable effective antisense sequences prior to cell-based screening of oligonucleotides (Zuker et al., 1999; Ding and Lawrence, 2001, 2003; Matveeva et al., 2003; Ding et al., 2004; Mathews et al., 2004). This approach, however, suffers drawback that the predictions may not truly reflect the actual scenario in the cell (Mathews et al., 1999; Kurreck et al., 2003).

It has been reported that there are several motifs that are associated with nonantisense effects. ASOs that are G-rich are well known for their nonantisense effects, which have been attributed to the tendency of these oligonucleotides to form G-quartet structures that then interfere with biological processes (Ecker et al., 1993; Bennett et al., 1994; Wyatt and Stein, 1999). Homopolymers of other sequences also form unusual structures and draw concerns on association with nonantisense activity (Freier et al., 2001). In addition, Kreig et al. (1994) reported that oligonucleotides containing CG motifs induce immunostimulatory effects. Further, an oligonucleotide containing

GGC motif was reported to exhibit nonantisense activity by inhibiting enzymatic activity of tyrosine kinase $p210^{bcr\text{-}abl}$ (Bergan et al., 1994, 1995). In contrast to these "bad motifs", Tu et al. (1998) reported that the tetranucleotide motif, TCCC is associated with antisense activity. Therefore, when designing oligonucleotides for antisense experiment, it is important to exclude oligonucleotides containing those "bad" motifs and include oligonucleotides containing TCCC motif to improve the "hit rate" for antisense discovery and avoid screening oligonucleotides likely to have undesirable nonantisense effects (Freier et al., 2001; Stein et al., 2001).

1.2.4.2.In vitro cellular uptake of ASOs

In cultured cells, internalization of ASOs is generally extremely inefficient. The polyanionic nature of ASOs is the primary cause of their inadequate and inefficient cellular association, owing to electrostatic repulsion from the negatively charged cell surface. It is generally believed that ASOs that are associated with the cell membrane are taken up by cells by a combination of receptor-mediated (at low concentrations) and fluid-phase (at high concentrations) endocytic mechanisms (Loke et al., 1989; Beltinger et al., 1995; Nakai et al., 1996). This small fraction of ASOs that does obtain cellular access is susceptible to inactivation and degradation in the endosomes. Upon endocytic internalization, ASOs are compartmentalized into endosomal vesicles, where ASOs can be inactivated or degraded. The endosome undergoes acidification to a pH of 5 to 6, which in addition to promoting acidic hyrolysis, activates lysosomal enzymes that can rapidly degrade ASOs (Akhtar and Juliano, 1992; Holmes et al., 1999; Wu-Pong et al., 2000). Only a small fraction of the ASOs escapes from the endosomes and enters the cytoplasm and nucleus to exert their actions on the target mRNA (Hughes et al., 2001; Liu and Huang, 2002).

A number of methods have therefore been developed to facilitate in vitro ASO cellular uptake (Hughes et al., 2001; Liang et al., 2002). By far the most commonly used delivery system is cationic lipids and many proprietary formulations such as Lipofectamine, Lipofectin (Invitrogen, Carlsbad, CA, USA), Effectene (Qiagen, Valencia, CA, USA) and Transfectam (Promega Corporation, Madison, WI, USA) are commercially available (Filion and Phillips, 1997; Kurreck J et al., 2003). Cationic lipids form complexes with ASOs as a result of opposing charges. This formation imparts an overall postive charge and facilitates attachment of the lipid-ASO complex to the anionic cell membrane. The complex is taken up by endocytosis but once inside an endosomal vesicle, the cationic lipid destablilizes the endosomal membrane and helps to set the ASO free into the cytoplasm to hybridize to its target mRNA (Gewirtz et al., 1998). Despite the appreciable success of cationic lipids in ASO transfer, studies showed that different cell types show variations in their sensitivity to cationic lipid-mediated ASO delivery and cells of lymphoid origin were found to be ineffectively transfected using this class of agents (Potter et al., 1984; Giles et al., 1998; Kronenwett et al., 1998; Hill et al., 1999). As such, cationic lipids are not universally applicable for all in vitro applications and therefore other approaches should be used for these cell types.

Electroporation is a delivery method that is applicable to all cell types, even those such as lymphocytes, which cannot be transfected with other methods such as cationic lipids or calcium phosphate (Potter et al., 1984). Electroporation makes use of the fact that the cell membrane acts as an electrical capacitor that is unable to pass current. Subjecting cells to a high-voltage electric field results in the temporary breakdown of their membranes and the formation of pores that allow ASOs to enter into the

cytoplasm and nucleus and exert their actions on target mRNA. Electroporation is widely used because of several desirable properties. First, it yields a high transfection efficiency. In addition, commercial apparatuses that are safe and easy to use are available. Further, it gives extremely reproducible results. However, this technique suffers drawbacks of high cell death and requires more cells and ASOs than other delivery systems (Potter et al., 1984; Bergan et al., 1996; Flanagan and Wagner, 1997).

1.2.4.3.Important controls for antisense experiments

Controls are important in antisense experiments to prove that any observed effect is due to a specific antisense knockdown of the target mRNA. A number of suggestions have been proposed. Briefly, at the very least, one should (1) demonstrate a reduction in targeted mRNA and/or protein, (2) determine the effects of the "active" oligonucleotide on gene products of closely related isotypes, and (3) determine the effect of scrambled or mismatched oligonucleotide on the targeted gene product. These simple rules ensure that the chances of misinterpreting data are minimized (Stein and Kreig, 1994; Kurreck et al., 2003).

1.3. Project objectives and approach

PLCγ1 plays an important role in T cell activation and thus serves as a valuable target for molecular intervention. Therefore, in this present study, we identified and characterized an ASO to PLCγ1 as an alternative approach to interrupt T cell activation.

In this study, software such as *mfold*, OLIGO 6, Sfold and Oligo Walk which calculate and predict mRNA secondary structure, single-stranded regions in target mRNA secondary structure and hybridization thermodynamics (Zuker et al., 1999;

Ding and Lawrence, 2001, 2003; Matveeva et al., 2003; Ding et al., 2004; Mathews et al., 2004) were used to identify probable effective PLCγ1 antisense sequences prior to screening in EL4.IL-2, a mouse T cell line. Special consideration was given to the design of the oligonucleotides to avoid nonantisense effects (Ecker et al., 1993; Bennett et al., 1994; Bergan et al., 1994; Kreig et al., 1994; Bergan et al., 1995; Wyatt and Stein, 1999; Freier et al., 2001). Selected oligonucleotides were full-backbone phosphorothioate-modified to increase their nuclease resistance and promote RNase H activity (Gewirtz et al., 1998; Cooper et al., 1999; Crooke et al., 1999; Roth and Yarmush, 1999). PS-modified oligonucleotides were then introduced into EL4.IL-2 cells by electroporation and antisense efficacy was assessed by quantitating PLCγ1 mRNA and protein levels. Effects of PLCγ1 protein downregulation on IP_3 and intracellular calcium levels in anti-CD3/CD28-stimulated EL4.IL-2 cells were further investigated. Finally, the functional consequence of antisense downregulation of PLCγ1 on anti-CD3/CD28-mediated IL-2 production and T cell proliferation were assessed *in vitro*.

2. MATERIALS AND METHODS

2.1. Materials

The following materials were used in this study: ASOs, scrambled control oligonucleotide, PLCγ1 primers and PLCγ2 primers (Sigma-Proligo, Boulder, CO, USA); murine lymphoma cell line EL4.IL-2 (American Type Culture Collection, Manassas, VA, USA); RPMI 1640 medium, L-glutamine, penicillin, streptomycin, OptiMEM, TRIZOL reagent, Hanks' balanced salt solution (HBSS), Fura-2/acetoxymethylester (fura-2/AM) and pluronic F127 (Invitrogen, Carlsbad, CA, USA); fetal bovine serum (FBS; Hyclone, South Logan, UT, Africa); Na_3VO_4, NaF, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, bovine serum albumin (BSA), 2-mercaptoethanol, mouse anti-β-actin monoclonal antibody (mAb), diethylpyrocarbonate (DEPC), probenecid, ionomycin and perchloric acid (PCA) (Sigma-Aldrich, St. Louis, MO, USA); ammonium persulfate (APS), 30% acrylamide/Bis solution (37.5:1), tetramethylethylenediamine (TEMED), AP conjugate substrate kit, agarose and TMB peroxidase EIA substrate kit (Bio-Rad Laboratories, Hercules, CA, USA); bicinchoninic acid (BCA) protein assay and Restore western blot stripping buffer (Pierce Biotechnology, Inc., Rockford, IL); bromophenol blue (Merk & Co., Inc., Whitehouse Station, NJ, USA); Tween-20 (Duchefa Biochemie B.V., Haarlem, The Netherlands); mouse anti-PLCγ1 mAb, hamster anti-CD3 mAb, hamster anti-CD28 mAb and mouse IL-2 BD OptiEIA enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody and alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG antibody (DakoCytomation Denmark A/S, Glostrup, Denmark); enhanced chemiluminescence (ECL) and western blotting detection reagents (Amersham Biosciences, Buckinghamshire, England); oligo$(dT)_{15}$ primer, dNTPs, AMV reverse transcriptase,

Rnasin ribonuclease inhibitor, PCR master mix, β–actin primers and CellTiter 96 AQ_{ueous} One Solution assay (Promega Corporation, Madison, WI, USA); HitHunter IP_3 fluorescence polarization assay (DiscoveRx, Fremont, California).

2.2. Design and synthesis of ASOs

In this study, ASOs targeting at mouse PLCγ1 mRNA were designed using 2 different approaches (design approach A and design approach B) and are shown in Table 2. In both approaches, mouse PLCγ1 mRNA sequence (accession number NM 021280) was obtained from Genbank. Designed ASO sequences and scrambled control oligonucleotide were synthesized as full-backbone PS-modified oligonucleotides to increase their resistance to nuclease degradation and promote RNase H-mediated mRNA breakdown (Gewirtz et al., 1998; Cooper et al., 1999; Crooke et al., 1999; Roth and Yarmush, 1999).

2.2.1. Design approach A

ASO1 and ASO2 were designed using design approach A. PLCγ1 mRNA secondary structure was predicted using the *mfold* software, version 3.1.2 (http://bioweb.Pasteur.fr/seqanal/interfaces/mfold-simple.html; Zuker et al., 1999), which predicts optimal and suboptimal secondary structures for an RNA molecule. OLIGO 6.0 (http://www.oligo.net/oligo.htm; Molecular Biology Insights, Inc., CO, USA) was used to select oligonucleotides that do not form inter- and intra-molecular oligonucleotide self structures. NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/) was used to align ASO sequence to NCBI sequence databases to ensure ASO designed is homologous only with target mRNA and not with other mRNA.

Table 2. Oligonucleotides used in this study

Oligonucleotide	Target Position (ORF)	Sequence	Design Approach
ASO1	511 - 530	5′-CATGTTCACTTCATCCTCAG-3′	A
ASO2	1490 - 1509	5′-TCTTCCTCCTGAGCTGGTTG-3′	A
ASO3	2632 -2651	5′-CAGCTGCTTCTTCCCACCAT-3′	B
ASO4	4015 - 4034	5′-GCACTTCCACAGCTTGTCAT-3′	B
Scrambled control	-	5′-TACCACCCTTCTTCGTCGAC-3′	-

Scrambled control share the same base composition with ASO3 but has a reversed order.
ORF = open reading frame.

ASOs were designed according to the following principles: (1) complementarity to single-stranded region in the predicted mRNA secondary structure, (2) avoidance of homopolyers and GGC motifs that support nonantisense effects, (3) minimal cytosine-guanine motifs, (4) less than 50% guanine content, (5) no formation of inter- and intra-molecular oligonucleotide self structures, (6) no homology with other mRNA, and (7) preferable inclusion of good motif, TCCC which is associated with strong antisense activity (Neckers and Iyer, 1998; Freier et al., 2001).

2.2.2. Design approach B

ASO3 and ASO4 were designed using design approach B. Oligo Walk program from the package RNAstructure 4.2 (http://128.151.176.70/RNAstructure.html; Mathews et al., 2004) was used to predict the equilibrium affinity of complementary oligonucleotides to PLCγ1 mRNA by calculating free energies ($\Delta G°_{37}$) for (1) oligonucleotide-mRNA duplex ($\Delta G°_{37duplex}$), (2) inter-oligonucleotide pairing, and (3) intra-molecular oligonucleotide pairing. Sfold (http://sfold.wadsworth.org; Ding and Lawrence, 2001, 2003; Ding et al., 2004) was used to predict probable PLCγ1 mRNA secondary structures and PLCγ1 mRNA accessibility sites to ASO. NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/) was used to align ASO sequence to NCBI sequence databases to ensure ASO designed is homologous only with target mRNA and not with other mRNA.

ASOs were designed according to the following principles: (1) formation of stable duplexes with PLCγ1 mRNA ($\Delta G°_{37\ duplex} \leq$ -30 kcal/mol), (2) formation of less stable self-structures ($\Delta G°_{37}$ for inter-oligonucleotide pairing $\geq$ -8 kcal/mol and $\Delta G°_{37}$ for intra-molecular pairing $\geq$ 1.1) [Matveeva et al., 2003], (3) complementarity to sites on

PLCγ1 mRNA which are predicted by Sfold to have a probability of being single-stranded of ≥ 0.6 (Ding and Lawrence, 2001), (4) avoidance of homopolyers and GGC motifs that support nonantisense effects, (5) minimal cytosine-guanine motifs, (6) less than 50% guanine content, (7) no homology with other mRNA, and (8) preferable inclusion of good motif, TCCC (Neckers and Iyer, 1998; Freier et al., 2001).

2.3. Cell culture

EL4.IL-2, a lymphoma cell line derived from C57BL/6 mouse was grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were maintained in logarithmic phase of growth by subculturing four times weekly.

2.4. Transfection by electroporation

ASOs were transfected into EL4.IL-2 cells and screened for their ability to inhibit PLCγ1 expression. An electroporation transfection protocol was employed because lymphocytes cannot be transfected with other procedures (e.g., calcium phosphate or cationic lipids) (Potter et al., 1984; Giles et al., 1998; Kronenwett et al., 1998; Hill et al., 1999). EL4.IL-2 cells were washed once in OptiMEM and resuspended at 1×10^7 cells/ml. The cell suspension was combined with the indicated concentrations of ASO and incubated for 5 min at room temperature. The mixture was transferred to a 4-mm gap electroporation cuvette and cells were permeabilized with a single pulse from BTX Electro Cell Manipulator 630 (Harvard Bioscience, Holliston, MA, USA) set at 250 V, 25 ohms and 1000 μF. These conditions were defined as producing optimal permeabilization with minimal toxicity in preliminary experiments. The cells were

incubated for 10 min in the electroporation cuvette and then transferred to 10 ml of pre-warmed gassed culture medium in 100 mm cell culture dishes (BD Biosciences, San Jose, CA, USA) and maintained under their normal culture conditions. Cells were harvested for protein or mRNA analysis at the defined time intervals.

2.5. Gel electrophoresis and Western blot analysis

Cells were harvested in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM Na_3VO_4, 34 mM NaF, 5 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin] and lysed on ice for 30 min before centrifugation at 4°C, 10000 g for 5 min. The supernatants were collected and assayed for protein concentrations using BCA protein assay reagents and BSA of known concentrations as the standard. The supernatants were then mixed with 5X sample buffer [312.5 mM Tris-HCl (pH 6.8), 50% glycerol, 25% 2-mercaptoethanol, 10% SDS and 0.0625% bromophenol blue] and boiled for 5 min. Proteins (10 μg per lane) were separated according to size by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% SDS-polyacrylamide gel and then transferred onto a polyvinylidene fluoride (PVDF; Bio-Rad Laboratories, Hercules, CA, USA) membrane using a semi-dry transblotter (ATTO Corp., Tokyo, Japan). The membrane was blocked in Tween 20-Tris-buffered saline [TTBS; 0.05% Tween 20, 0.1 M Tris-HCl (pH 7.5) and 0.9% NaCl] containing 5% nonfat dried milk for 1 h at room temperature with agitation and then probed with mouse anti-PLCγ1 antibody diluted in TTBS (1:10000) containing 1% nonfat dried milk overnight at 4°C. The membrane was washed extensively in TTBS and then incubated 1 h at room temperature with HRP-conjugated goat anti-mouse IgG antibody diluted 1:8000 in TTBS containing 1% nonfat dried milk. After extensive washes, the membrane was incubated with ECL western blotting detection

reagents according to the manufacturer's protocol. The ECL-treated membrane was further exposed to autoradiography film (Amersham Biosciences, Buckinghamshire, England) to allow visualization of the PLCγ1 protein band. To confirm equivalent loadings among samples, the membrane was stripped and reprobed with mouse anti-β-actin antibody diluted 1:5000, followed by AP-conjugated rabbit anti-mouse IgG antibody (diluted 1:4000), and developed colorimetrically using AP conjugate substrate kit. PLCγ1 protein was quantitated relative to β-actin using Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD).

2.6. Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Cells were harvested into TRIZOL reagent and total RNA was isolated from the cell lysate according to the manufacturer's instructions. Briefly, cell lysate was mixed with chloroform and separated into an aqueous phase and an organic phase by centrifugation. The aqueous phase containing RNA was collected and RNA was precipitated with isopropyl alcohol. RNA pellet was then washed with 75% ethanol and resuspended in DEPC-treated Milli-Q water. Absorbance at 260 nm of the diluted RNA sample was determined spectrophotometrically and RNA concentration was calculated (one A_{260} unit equals 40 ng of single-stranded RNA/μl).

First strand complementary DNA (cDNA) was synthesized using a two-step reverse transcription protocol. Firstly, total RNA (1 μg) was incubated with oligo$(dT)_{15}$ primer (0.5 μg) for 10 min at 70°C. The mixture was then chilled on ice for 5 min. Next, AMV reverse transcriptase 1X buffer, dNTPs (0.5 mM), AMV reverse transcriptase (15 U) and Rnasin ribonuclease inhibitor (20 U) were added to the

mixture and incubated for 60 min at 42°C. Finally, the mixture was incubated on ice for 5 min to terminate the reaction.

PCR was carried out in a thermal cycler (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA). The reaction mixture contained 1X PCR master mix, 0.4 μM of each specific primer and 1 μl of cDNA in a final volume of 25 μl. Primers specific for mouse PLCγ1, PLCγ2 and β–actin were used (see Table 3). PLCγ1 and PLCγ2 primers were designed using the programs Prime (http://bioportal.bic.nus.edu.sg/gcg/prime.html; Accelrys, Inc., San Diego, CA, USA) and OLIGO 6.0 (http://www.oligo.net/oligo.htm; Molecular Biology Insights, Inc., CO, USA). They were designed according to the following principles: (1) 20-26 nucleotides, (2) 40-60% GC content, (3) avoidance of complementary sequences within primer, (4) avoidance of complementary sequences between primers, (5) avoidance runs of 3 or more G or C at the 3'-end, and (6) avoidance of a T or an A at the 3' end. The resulting PCR products were separated and visualized on an ethidium bromide-stained agarose gel. PLCγ1 and PLCγ2 mRNA levels were quantitated by Gel-Pro imaging software and normalized by reference to actin.

2.7. IP_3 fluorescence polarization assay

At 24 h posttransfection with PLCγ1 ASO, cells were harvested and IP_3 levels following T cell receptor ligation were quantitated using the HitHunter IP_3 fluorescence polarization assay according to the manufacturer's recommendations. This assay is based on competitive binding between an IP_3 fluorescent tracer and unlabelled IP_3 from cell lysates for IP_3 binding protein (Figure 10). Bound IP_3 tracer is restricted from rotating and will "tumble" more slowly in solution, creating a

Table 3. Primer sequences for RT-PCR analysis

Gene name	Sequences	Product size (bp)	PCR program
PLCγ1	Forward primer: 5′-CGGAAACCAAGGCTGAGAAGTATG-3′	402	Denaturation 94°C, 30 sec Annealing 60°C, 30 sec Extension 72°C, 30 sec
	Reverse primer: 5′-CAGCCACCTCAATCTCCACAAAAG-3′		
PLCγ2	Forward primer: 5′-CAATGAGAGAGACCGCAGAG-3′	387	
	Reverse primer: 5′-TAGACAAGATGACGGGGAAG-3′		
β-actin	Forward primer: 5′-TCATGAAGTGTGACGTTGACATCCGT-3′	285	
	Reverse primer: 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′		

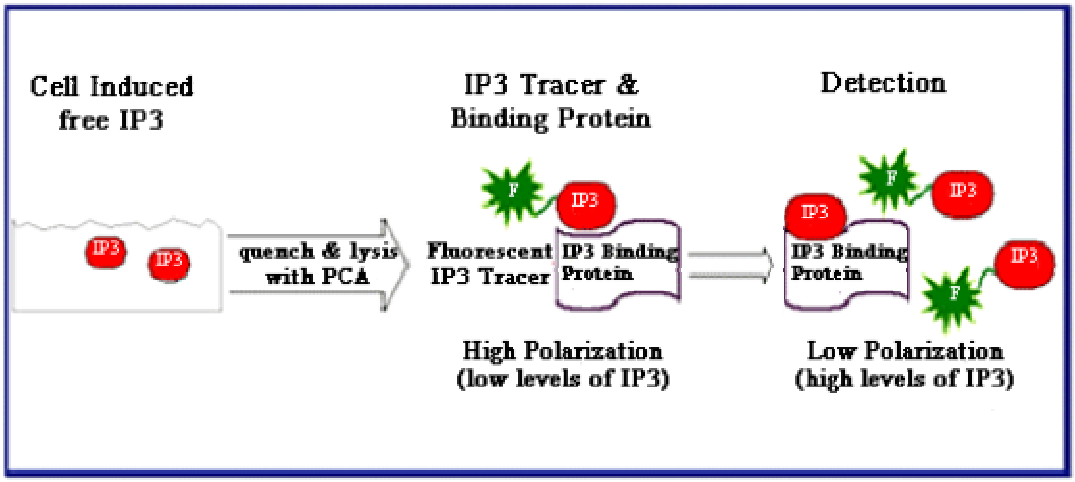

Figure 10. HitHunter IP_3 fluorescence polarization assay principle (adapted from www.discoverx.com)

polarized signal (high mP). Free IP_3 from cell lysates compete for binding to the IP_3 binding protein and allows the IP_3 tracer to rotate freely upon excitation with plane polarized light. Thus, the polarized signal is inversely proportional to the amount of IP_3 in the cell lysates. Briefly, cells were washed twice and suspended in phosphate-buffered saline (PBS; pH 7.3, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$ and 1.4 mM KH_2PO_4). Cells (8×10^4) were transferred to a 96-well flat-bottom, black microtiter plate (Greiner Bio-One, Frickenhausen, Germany) and stimulated with 3 μg/ml anti-CD3 and 10 μg/ml anti-CD28 for 10 min at 37°C. At the end of incubation, induced cells were quenched and lysed by the addition of PCA at a final concentration of 0.05 N. Fluorescent IP_3 tracer and IP_3 binding protein were subsequently added and mixed thoroughly on a plate shaker for 5 min. IP_3 levels in the sample were determined by measuring the fluorescence polarization signal using a BMG FLUOstar OPTIMA microplate reader (BMG LABTECH GmbH, Offenburg, Germany) with excitation wavelength set at 485nm and emission wavelength set at 530 nm.

2.8. Intracellular Ca^{2+} measurement

Cells were harvested 24 h after transfection with PLCγ1 ASO and changes in intracellular Ca^{2+} concentration following stimulation with hamster anti-CD3 and anti-CD28 mAbs were evaluated. Cells (2×10^6) were washed twice and suspended in HBSS (without calcium and magnesium and phenol red) containing 5% FBS, 20 mM HEPES and 2.5 mM probenecid. Cells were then loaded with the intracellular calcium indicator dye, Fura-2/AM (3 μM), in the presence of 0.01% Pluronic F127 for 30 min at 37°C in the dark. At the end of incubation, extracellular dye was removed by washing and cells were resuspended in HBSS containing calcium, magnesium, 5%

FBS, 20 mM HEPES and 2.5 mM probenecid. Anti-CD3 mAb (3 μg/ml) was added to the cell suspension and incubated for 1 h on ice. Unbound antibody was removed by washing. Cells were resuspended at a final density of 1×10^6 cells/ml and kept at 37°C until analysis. Cells were transferred to a cuvette and stimulation started with the addition of soluble anti-CD28 mAb (10 μg/ml). Changes in intracellular Ca^{2+} concentration were determined by monitoring fluorescence at 340 nm and 380 nm using an InCyt CV2 dual-wavelength fluorescence photometry system (Intracellular Imaging, Inc., Cincinnati, Ohio). Ionomycin (1 μM) stimulation was performed to evaluate cell loading with the Fura-2 indicator.

2.9. IL-2 measurement by enzyme-linked immunosorbent assay (ELISA)

Cells (2×10^4) were stimulated with both anti-CD3 (0.125 μg/ml) and anti-CD28 (0.25 μg/ml) mAbs in triplicate wells at 12 h posttransfection with PLCγ1 ASO. The culture supernatants were collected 24 h later and IL-2 concentration was quantitated using commercially available mouse IL-2 ELISA kit according to the manufacturer's instructions. Firstly, purified anti-mouse IL-2 capture mAb was diluted 1:250 in the coating buffer (0.1 M carbonate, pH 9.5). Diluted capture antibody (50 μl per well) was used to coat a 96-well Nunc Maxisorp ELISA plate (eBioscience, San Diego, CA, USA), which was sealed and incubated overnight at 4°C. Prior to adding samples, the plate was washed and blocked with PBS containing 10% heat-inactivated FBS for 2 h at room temperature. The standard, recombinant mouse IL-2 was diluted to the concentrations of 800, 400, 200, 100, 50, 25, 12.5, 6.3 and 3.1 pg/ml. After washing, standards and cell culture supernatants (50 μl per well) were added to the coated plate and incubated for 2 h at room temperature. After extensive washes, working detector (containing biotinylated anti-mouse IL-2 detection antibody and avidin-HRP

conjugate diluted 1:250 in PBS/10% heat-inactivated FBS) was added to the plate (50 μl per well) and incubated for 1 h in the dark. The plate was extensively washed and final detection was achieved through the addition of the substrate solution containing TMB and hydrogen peroxide. Color reaction was stopped after 30 min by the addition of 1 N H_2SO_4. PBS/0.05% Tween-20 was used as washing buffer between steps. Optical densities were read at 450 nm (with wavelength correction at 570 nm) on a Tecan Sunrise microplate reader (Tecan Trading AG, Zurich, Switzerland). A standard curve was generated and the concentration of IL-2 in each sample was determined from the curve.

2.10. Proliferation assay

Anti-CD3/CD28-induced proliferation was assayed using the CellTiter 96 AQ_{ueous} One Solution assay according to the manufacturer's protocol. This assay is a colorimetric method for determining the number of viable cells in proliferation assays. The assay reagent contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling reagent, phenazine ethosulfate (PES) which combines with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Thus, the quantity of formazan product (measured at an absorbance of 490 nm) is directly proportional to the number of living cells in culture. Briefly, 2×10^4 cells were stimulated with 0.125 μg/ml anti-CD3 and 0.25 μg/ml anti-CD28 in triplicate wells in a 96-well plate at 12 h posttransfection with PLCγ1 ASO. Cells were returned to culture and harvested 24 h later for proliferation assay. CellTiter 96 AQ_{ueous} One

Solution reagent (20 μl per well) was added to the cells and the plate was returned for a further 4-h culture. At the end of incubation, proliferation was quantified by recording the absorbance at 490 nm.

2.11. Statistical analysis

Data are expressed as mean ± SEM. One-way analysis of variance followed by a Tukey test was used to determine significant differences between treatment groups. SPSS software (SPSS Inc., Chicago, USA) was used for all statistical analysis.

3. RESULTS

3.1. Effects of PLCγ1 ASO on PLCγ1 mRNA and protein in EL4.IL-2 cell line

3.1.1. Screening of active PLCγ1 ASO

ASOs against murine PLCγ1 mRNA were designed following principles described in *Materials and Methods*. Figure 11 shows the predicted secondary structure of mouse PLCγ1 mRNA and the sites targeted by ASOs used in this study. The limited gene-walk with ASOs complementary to mouse PLCγ1 mRNA identified an active ASO, ASO3 capable of downregulating PLCγ1 mRNA and protein (Figure 12). EL4.IL-2 cells were electroporated at 250 V in the presence of 20 μM PLCγ1-selective ASOs 1-4 or scrambled control oligonucleotide, and harvested 24 h later for protein analysis. ASO3 showed the highest antisense activity among the other 3 ASOs and was chosen for further studies (Figure 12A). ASO3 significantly decreased PLCγ1 protein expression by 40% whereas the scrambled control oligonucleotide, which has the same base composition as ASO3 but in a reversed order, was without effect (Figure 12B). To investigate whether ASO3 downregulate PLCγ1 at the mRNA level, cells were treated with 20 μM ASO3 for 6 h and harvested for mRNA analysis by RT-PCR. ASO3 was observed to markedly reduce PLCγ1 mRNA by 40% as well whereas the scrambled control had no significant effect (Figure 12C), suggesting that ASO3 exerted its antisense effects through an RNase H-mediated mechanism.

3.1.2. Concentration-response relationship of PLCγ1-selective ASO

Figure 13 depicts that ASO3 decreased PLCγ1 mRNA expression after electroporation into EL4.IL-2 cells in a concentration-dependent manner. In contrast, there was no significant target mRNA reduction in cells exposed to increasing concentrations of scrambled control oligonucleotide. ASO3 or scrambled control was

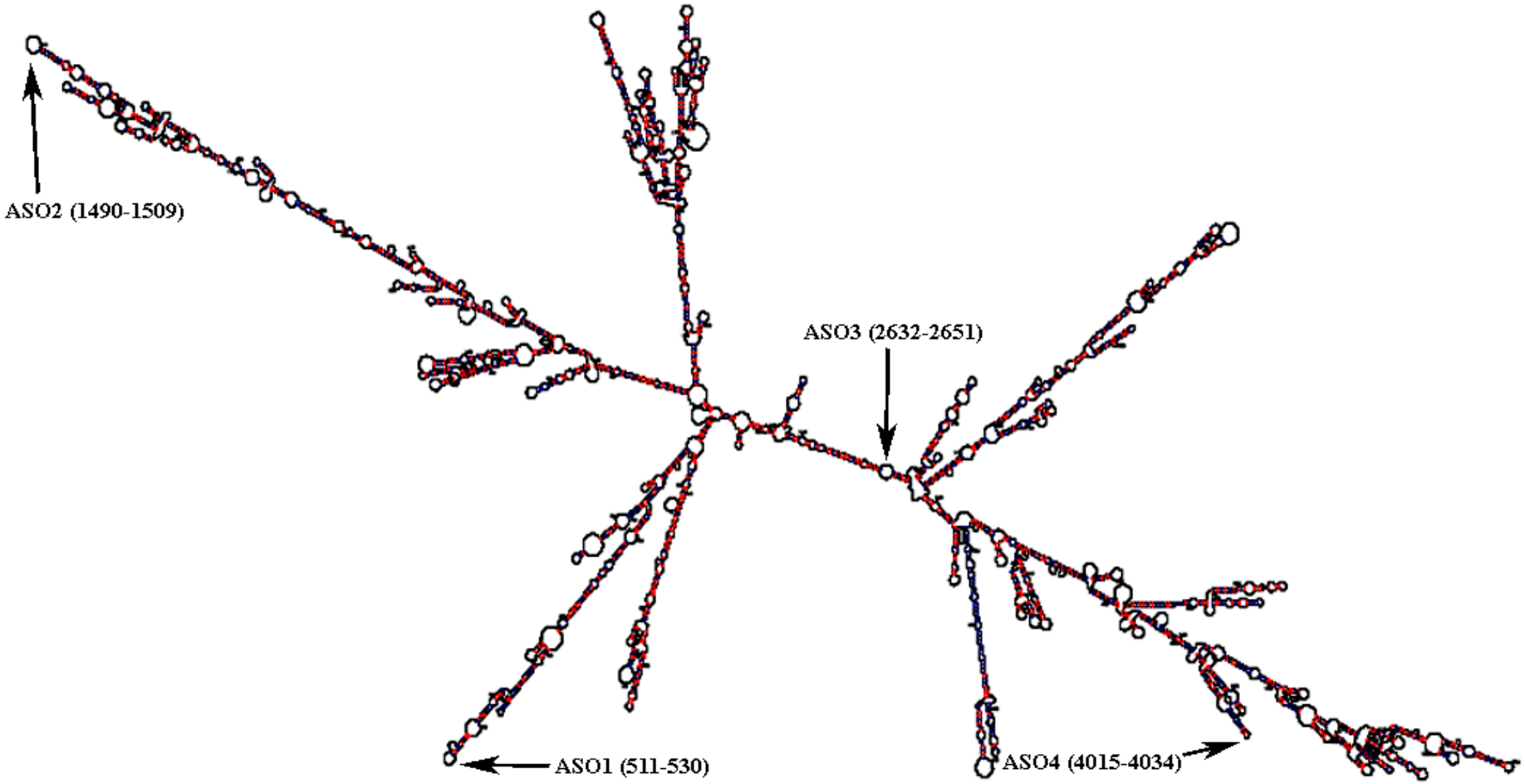

Figure 11. Predicted PLCγ1 mRNA secondary structure and sites targeted by ASOs used in this study.

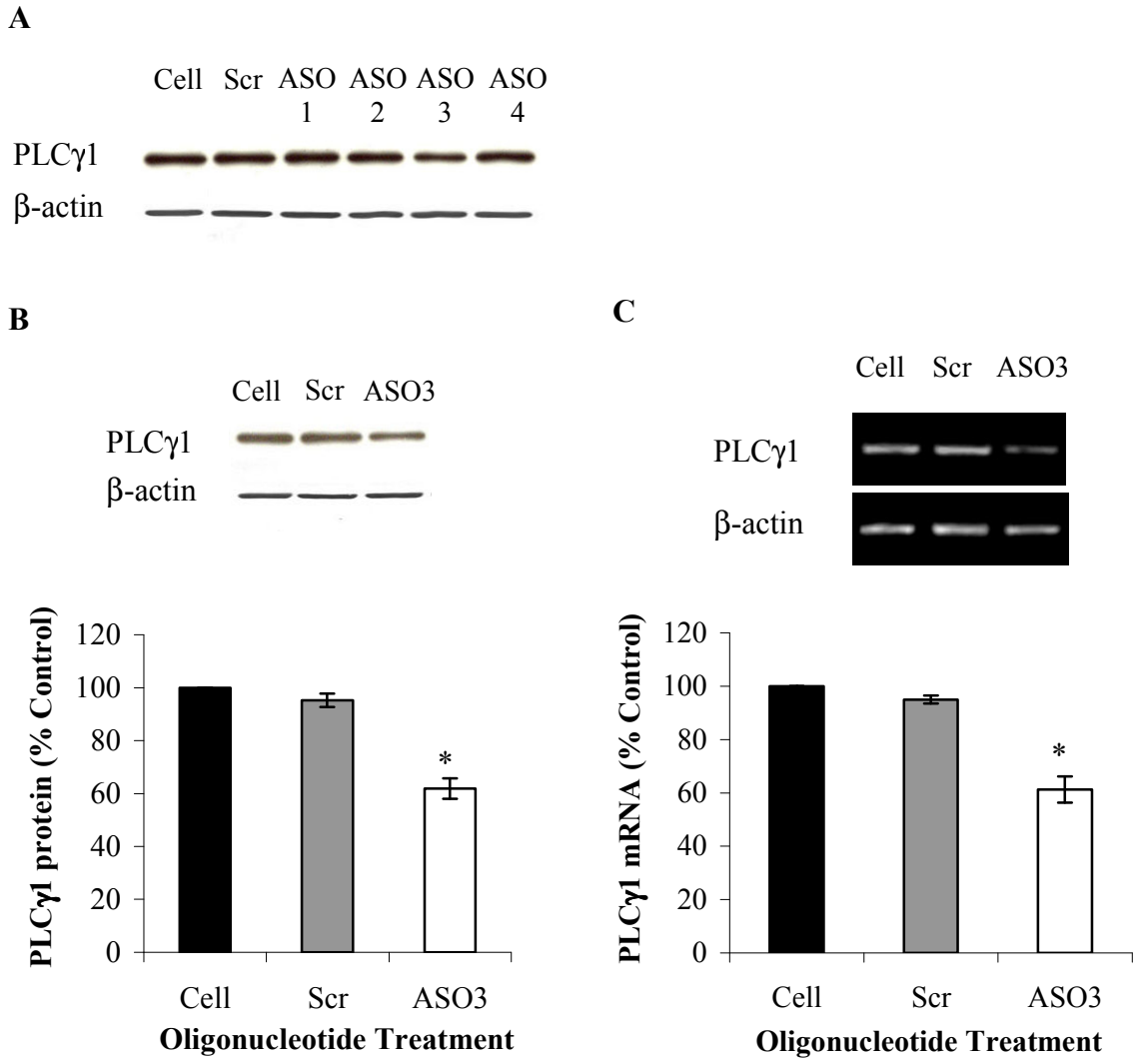

Figure 12. Initial screening of active PLCγ1 ASO. (A) Several ASOs targeting PLCγ1 mRNA were designed and tested for their ability to inhibit PLCγ1 protein expression. EL4.IL-2 cells were transfected with ASOs 1-4 (20 μM) by electroporation and harvested for protein analysis at 24 h posttransfection. (B) ASO3-mediated inhibition of PLCγ1 protein expression. *Top panel*, Western blot showing PLCγ1 downregulation in ASO3-treated cells. *Bottom panel*, quantitation of Western blot results (n=4). (C) Reduction of PLCγ1 mRNA by ASO3. *Top panel*, cells were treated with 20 μM PLCγ1 ASO or scrambled control oligonucleotide. Total RNA was harvested 6 h later and mRNA expression was measured by RT-PCR. *Bottom panel*, quantitation of RT-PCR results (n=4). Results were normalized to β-actin levels and expressed as a percentage of cell only control (mock electroporation without oligonucleotide). Scr = scrambled control oligonucleotide. * Significant difference from scrambled control oligonucleotide, $p < 0.01$.

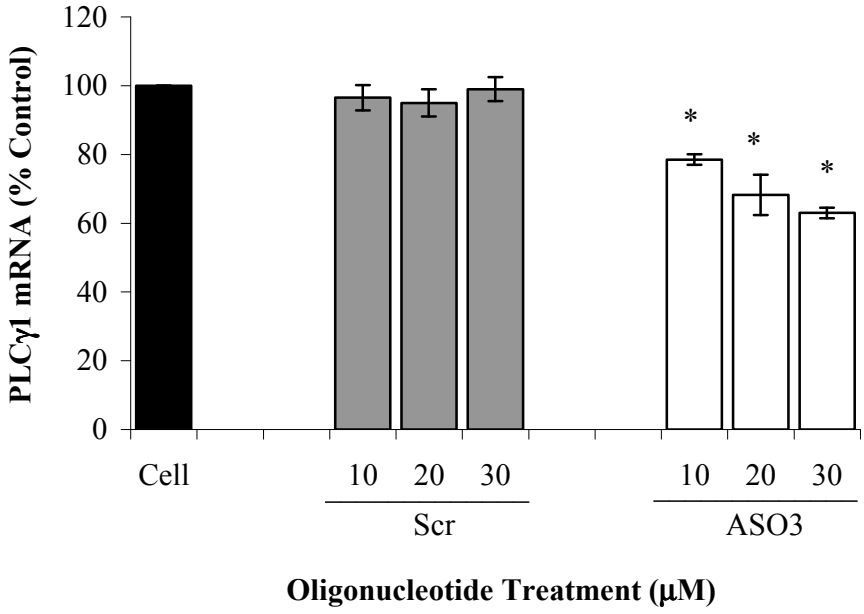

Figure 13. Concentration-dependent inhibition of PLCγ1 mRNA by ASO3. PLCγ1 mRNA levels in EL4.IL-2 cells 6 h after transfection with ASO3 or scrambled control at the indicated concentrations. PLCγ1 mRNA levels were normalized to β-actin mRNA levels and expressed as a percentage of cell only control. Values shown are the mean ± SEM of four separate experiments. * Significant difference from scrambled control oligonucleotide, $p < 0.01$.

used at concentrations ranging from 10 – 30 μM for RT-PCR analysis and ASO3-mediated inhibition was observed to reach a plateau above 20 μM of electroporated ASO3.

3.1.3. Time-course analysis of PLCγ1-selective ASO

EL4.IL-2 cells were treated with 20 μM ASO3 and harvested at the time points as indicated for PLCγ1 mRNA and protein analysis. Figure 14 shows the time-course profile of PLCγ1 mRNA and protein inhibition by ASO3 with the scrambled control having no effect on PLCγ1 expression. It was observed that ASO3-mediated PLCγ1 mRNA depletion preceded PLCγ1 protein reduction. Maximum downregulation of PLCγ1 mRNA and protein by ASO3 was seen 6 h and 24 h after electroporation respectively.

3.1.4. Specificity of PLCγ1-selective ASO

3.1.4.1.Cross-reactivity study

The activity of ASO3 in EL4.IL-2 cells was highly specific to PLCγ1. RT-PCR analysis of cells treated with ASO3, using PLCγ2 primers failed to demonstrate any change in PLCγ2 expression, indicating specificity of ASO3 for the γ1 isoform (Figure 15).

3.1.4.2.Scrambled control analysis

Figures 12 to 14 depict that ASO3-treated cells exhibit reduced PLCγ1 expression while scrambled control oligonucleotide-treated cells exhibit no change in PLCγ1 expression, suggesting ASO3 reduced PLCγ1 expression in a sequence-specific manner.

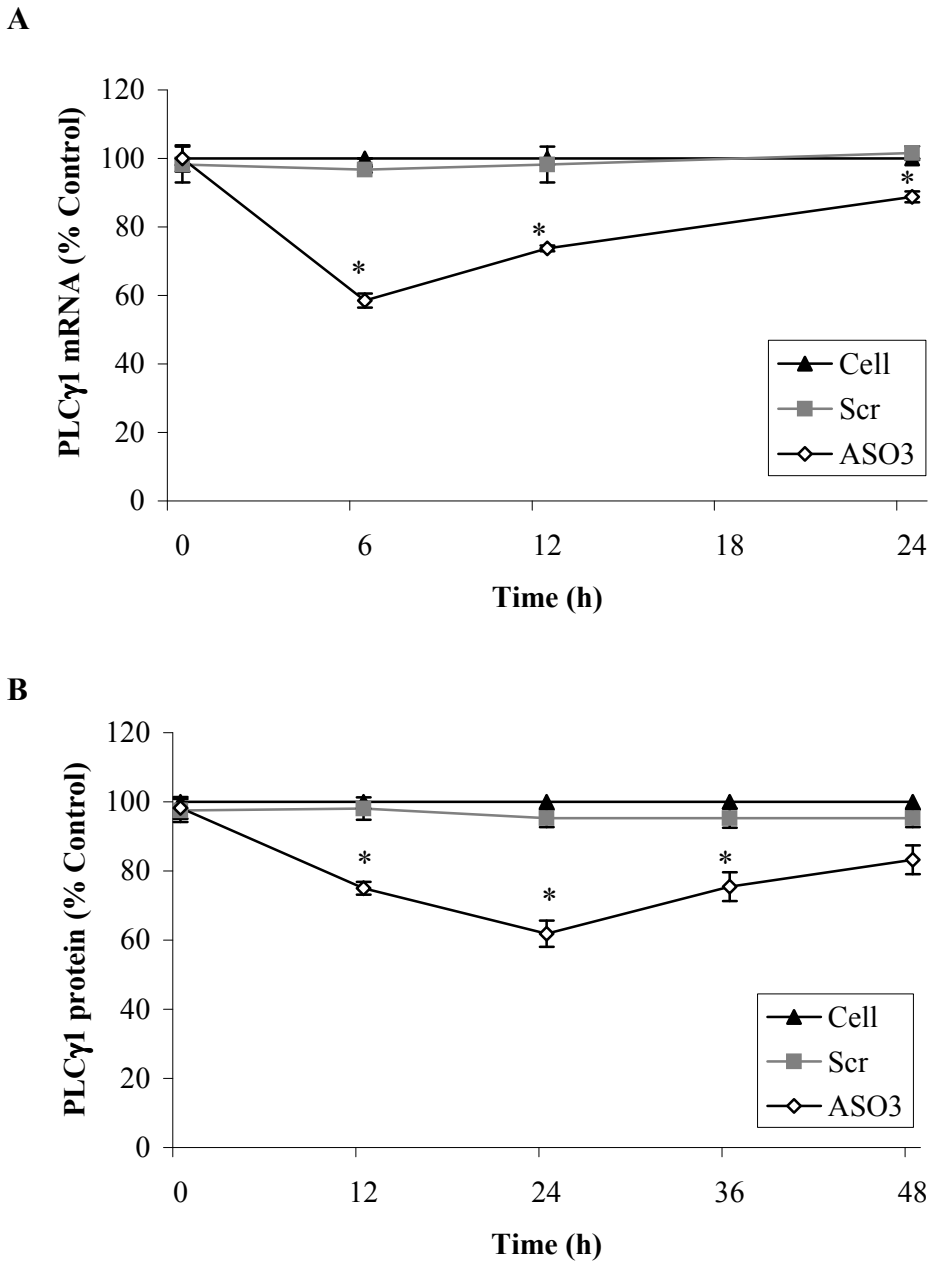

Figure 14. Time-course effect of ASO3 for the reduction of PLCγ1 mRNA (A) and protein (B) in EL4.IL-2 cells. ASO3 (20 μM) was introduced into EL4.IL-2 cells by electroporation at 250 V. Samples were harvested at the time points as indicated. PLCγ1 mRNA and protein expression were determined by RT-PCR and Western blot respectively. Results shown are representative of four independent experiments. Data were normalized to β-actin levels and expressed as a percentage of cell only control. * Significant difference from scrambled control oligonucleotide, $p < 0.01$.

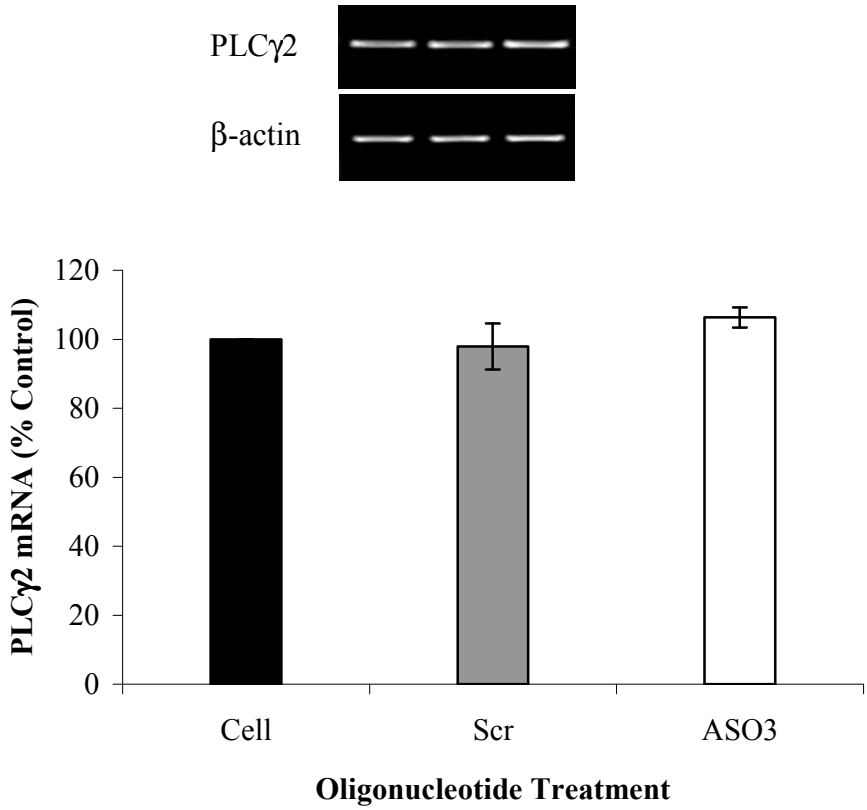

Figure 15. PLCγ1-selective ASO has no effect on PLCγ2 mRNA expression. Specificity of ASO3 was investigated by RT-PCR analysis of cells treated with 20 μM ASO3 using PLCγ2 primers. β-actin was used to normalize for sample loading. Results shown are representative of four independent experiments.

3.2. Effects of PLCγ1 protein downregulation on IP_3 and intracellular Ca^{2+} levels

With the identification of a highly active and specific PLCγ1 ASO, the effects of ASO3-mediated PLCγ1 protein downregulation on IP_3 and intracellular Ca^{2+} levels were investigated.

3.2.1. Effect of PLCγ1-selective ASO on anti-CD3/CD28-induced IP_3 generation

Figure 16 illustrates that PLCγ1-selective ASO significantly reduced IP_3 generation in EL4.IL-2 cells. Anti-CD3/CD28 stimulation of EL4.IL-2 results in activation of PLCγ1, which in turn, hydrolyzes PIP_2 into second messengers, DAG and IP_3. Pretreatment of cells with ASO3 before anti-CD3/CD28 stimulation significantly inhibited IP_3 generation by 30%. In contrast, scrambled control was unable to inhibit IP_3 generation.

3.2.2. Effect of PLCγ1-selective ASO on TCR-CD28 stimulation-induced intracellular Ca^{2+} increase

To study the effect of ASO3 on intracellular Ca^{2+} levels, cells were pretreated with ASO3 or scrambled control oligonucleotide for 24 h prior to stimulation with anti-CD3 and anti-CD28 antibodies. As shown in Figure 17, ASO3 significantly reduced the rise in intracellular Ca^{2+} triggered by TCR-CD28 costimulation whereas the scrambled control did not inhibit the Ca^{2+} increase.

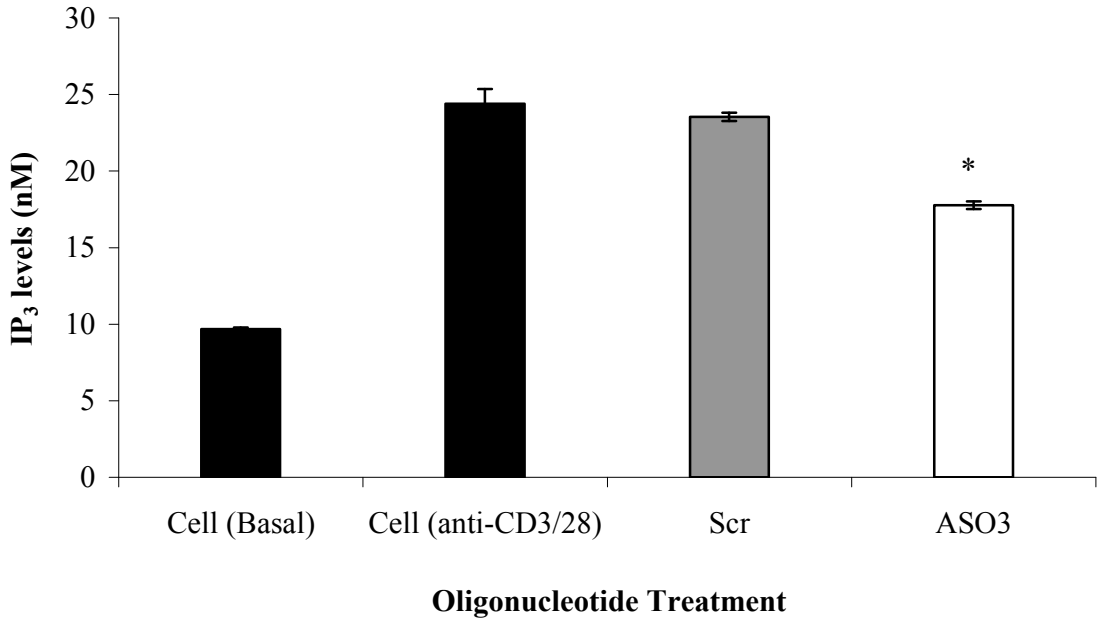

Figure 16. PLCγ1-selective ASO significantly reduced anti-CD3/CD28-induced IP_3 generation. EL4.IL-2 cells were treated with or without ASO3 (20 μM) for 24 h, followed by a 10-min stimulation with anti-CD3 and anti-CD28 antibodies. IP_3 generated was quantitated using IP_3 fluorescence polarization assay. Results shown are representative of four separate experiments. * Significant difference from scrambled control oligonucleotide, $p < 0.01$.

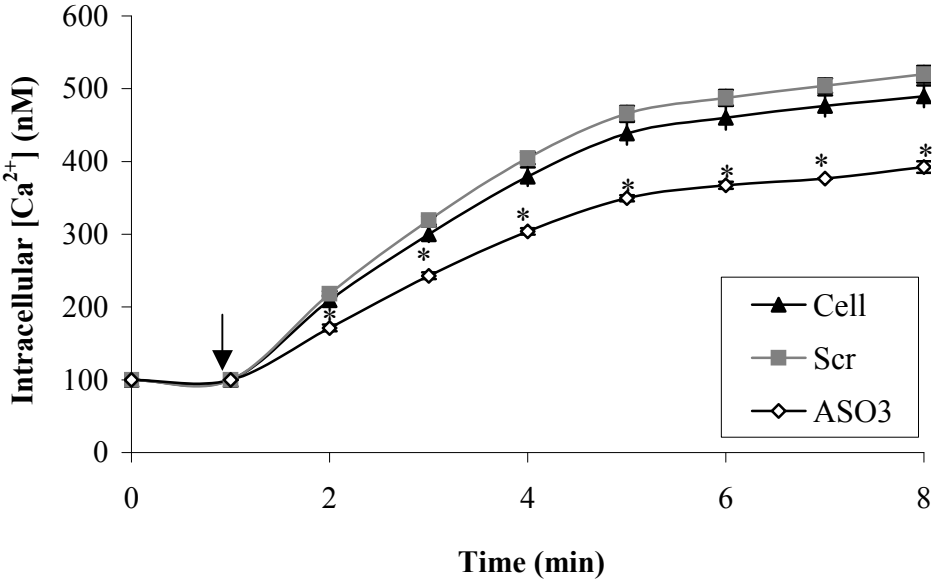

Figure 17. Significant reduction in anti-CD3/CD28-induced Ca^{2+} increase in ASO3-treated cells. PLCγ1-selective ASO or scrambled control oligonucleotide (20 μM) was introduced into EL4.IL-2 cells and incubated for 24 h. Cells were then loaded with Fura-2/AM and incubated with anti-CD3 for 1 h on ice. The arrow indicates the point of addition of anti-CD28. Data points represent the mean ± SEM of four separate experiments. * Significant difference from scrambled control oligonucleotide, $p < 0.01$.

3.3. Functional effects of PLCγ1 protein downregulation

To investigate the role played by PLCγ1 in T cells, the effects of ASO3-mediated PLCγ1 protein downregulation on IL-2 production and T cell proliferation were explored.

3.3.1. Effect of PLCγ1-selective ASO on IL-2 production

Figure 18 shows that ASO3 significantly reduced IL-2 production in EL4.IL-2 cells. Anti-CD3/CD28 costimulation of EL4.IL-2 cells results in a dramatic increase in IL-2 production. Pretreatment of cells with increasing concentrations of ASO3 (10 to 30 μM) before anti-CD3/CD28 stimulation significantly inhibited IL-2 production in a concentration-dependent manner. IL-2 inhibition was observed to reach a plateau above 20 μM of electroporated ASO3, similar to the trend observed in the concentration-dependent PLCγ1 mRNA downregulation study (Figure 13). To the contrary, cells exposed to scrambled control prior to costimulation did not exhibit any inhibitory effect on IL-2 production.

3.3.2. Effect of PLCγ1-selective ASO on T cell proliferation

To study the effect of ASO3 on T cell proliferation, cells were treated with ASO3 or scrambled control oligonucleotide for 12 h and stimulated with anti-CD3 and anti-CD28 antibodies for 24 h before they were harvested for proliferation assay. As shown in Figure 19, both ASO3 and, unexpectedly, scrambled control oligonucleotide significantly reduced proliferation of anti-CD3/CD28-simulated EL4.IL-2 cells.

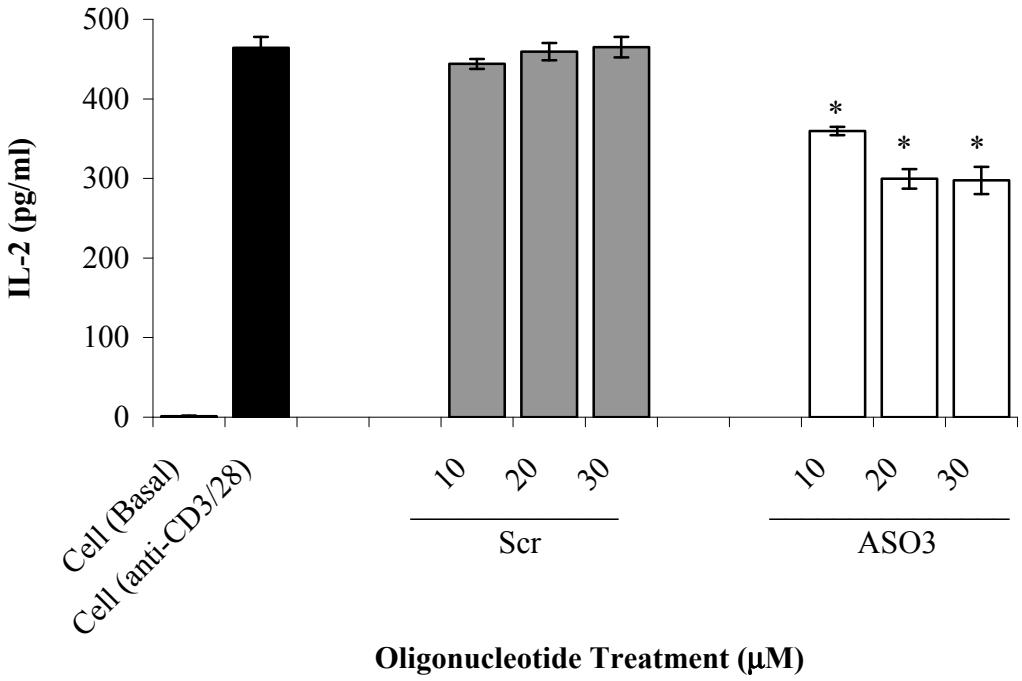

Figure 18. Concentration-dependent inhibition of IL-2 protein levels in EL4.IL-2 cell culture supernatants by PLCγ1-selective ASO. EL4.IL-2 cells were transfected with the indicated concentrations of ASO3 or scrambled control oligonucleotide and stimulated with anti-CD3/CD28 as described in *Materials and Methods*, after which supernatants were collected and IL-2 protein levels were analyzed by ELISA. Analyses shown are representative of four separate experiments performed in triplicates per treatment group. * Significant difference from scrambled control oligonucleotide, $p < 0.01$.

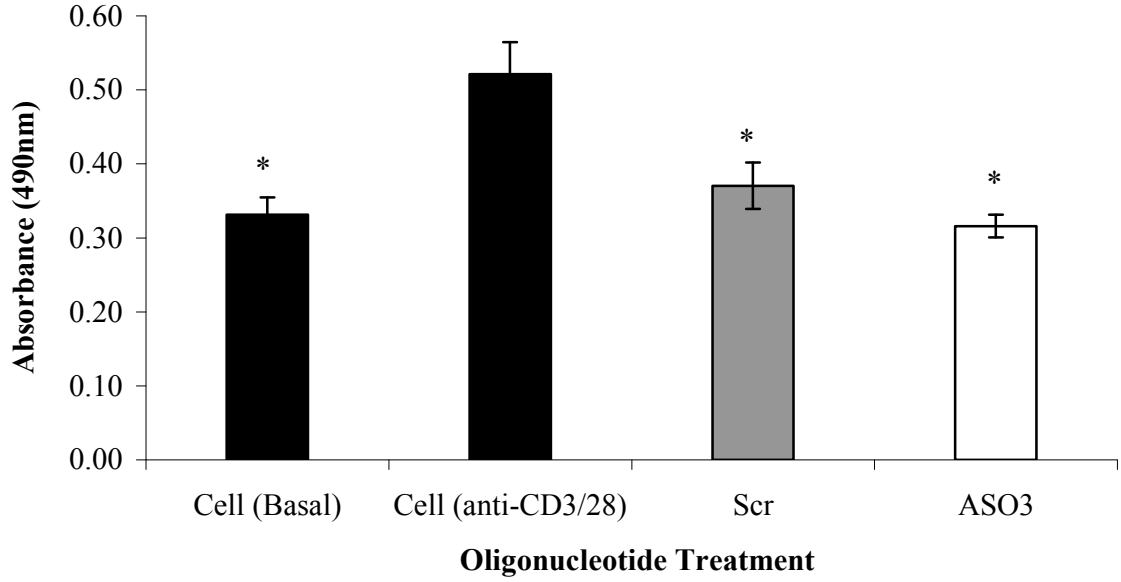

Figure 19. Inhibition of T cell proliferation by PLCγ1-selective ASO and scrambled control oligonucleotide. ASO3 or scrambled control oligonucleotide (20 μM) was introduced into EL4.IL-2 cells and incubated for 12 h. Cells were then stimulated with anti-CD3/CD28 for 24 h and harvested for proliferation assay. Data shown are the mean ± SEM of four independent experiments. * Significant difference from cell only control (mock-transfected control stimulated with anti-CD3/CD28), $p < 0.05$.

4. DISCUSSION

4.1. PLCγ1 – an attractive target for antisense inhibition of T cell activation

Upon antigen stimulation, T cells initiate a cascade of biochemical events that culminates in transcription of cytokine genes (including IL-2), cell proliferation, and acquisition of T-cell effector functions, in an effort to mount an immunological response against the antigen (Rao et al., 1997; Tomlinson et al., 2000).

TCR-mediated signaling is targeted by many immunosuppressive drugs in the prevention of GVHD and allograft rejection, and in the therapy for T cell-mediated autoimmune inflammatory conditions (Bierer et al., 1993; Xu et al., 1995; Goldman et al., 2000; Kang et al., 2003). Immunosuppressive agents such as hydroxychloroquine, cyclosporin A (CsA), tacrolimus (FK506), and glucocorticoids exert their effects by inhibiting TCR-induced signaling events such as intracellular calcium mobilization, calcineurin phosphatase activity, and AP-1, NFAT and NFkB transcriptional activity respectively (Barnes and Adcock, 1993; Bierer et al., 1993; Paliogianni et al., 1993; Xu et al., 1995; Goldman et al., 2000). However, these small molecules inhibitors are associated with several undesirable side effects and the drawback of resistant activated T cells (Goldman et al., 2000; Cristillo et al., 2003; Tsitoura and Rothman, 2004).

PLCγ1 is the predominant isoform in T cells and plays a prominent role in T cell activation (Wang et al., 2000; Wilde and Watson, 2001; Diaz-Flores et al., 2003). Interaction of T cell receptor with antigen results in activation of multiple protein kinases, which are in turn, linked to PLCγ1 activation (Baker et al., 2001). Activated PLCγ1 hydrolyzes PIP_2 to IP_3 and DAG, which in turn stimulate Ca^{2+} increase, PKC

activation and Ras activation (Kazanietz et al., 2000), all of which leads to the eventual activation of transcription factors regulating IL-2 gene expression and ultimately leading to T cell proliferation (Marais et al., 1993; Crabtree and Clipstone, 1994; Marais et al., 1998). Several studies supported that the downstream targets of PLCγ1 activation are important in T cell activation. Dornand and colleagues (1987) reported that lipoxygenase inhibitors suppressed IL-2 synthesis by inhibiting intracellular calcium increase and PKC activation by DAG. Further, Truneh and colleagues (1985) demonstrated the importance of Ca^{2+} and DAG in T cell activation by showing that a combination of Ca^{2+} ionophore and phorbol esters, which function as DAG analogues, could mimic TCR signals, leading to full T cell activation. On the basis of this knowledge, we adopted an antisense approach to selectively downregulate the synthesis of PLCγ1 as an alternative approach to interrupt T cell activation.

4.2. Identification of an active ASO targeting PLCγ1

ASOs targeted to murine PLCγ1 mRNA were designed following principles described in *Materials and Methods*. Two approaches were used in the antisense design, differing in the software being employed to aid in the prediction of probable efficient antisense sequences. In design approach A, the software *mfold* (http://bioweb.Pasteur.fr/seqanal/interfaces/mfold-simple.html; Zuker et al., 1999) was used to predict optimal and suboptimal secondary structures of mouse PLCγ1 mRNA. Single-stranded regions that were observed to appear in most of the predicted secondary structures were identified as probable hybridization-accessible sites on mouse PLCγ1 mRNA for ASO targeting. In addition, OLIGO 6.0 (http://www.oligo.net/oligo.htm; Molecular Biology Insights, Inc., CO, USA) was

used to select oligonucleotides that do not form inter- and intra-molecular oligonucleotide self structures. Design approach B, on the other hand, uses Sfold (http://sfold.wadsworth.org; Ding and Lawrence, 2001, 2003; Ding et al., 2004) to predict probable PLCγ1 mRNA secondary structures and antisense accessible sites on PLCγ1 mRNA. Oligo Walk program (http://128.151.176.70/RNAstructure.html; Mathews et al., 2004) was also used in this approach to predict the affinity of complementary oligonucleotides to PLCγ1 mRNA. Both of this software has been described by Ding et al (2001) and Matveeva et al. (2003) to greatly improve the "hit rate" in antisense screens. Unmodified phosphodiester oligonucleotides are rapidly degraded by nucleases and are not generally practical to use as antisense (Engels and Uhlmann, 2000). Hence, in this present study, we modified the backbone of the designed antisense sequences and scrambled control oligonucleotide with the most commonly used PS linkages to increase their resistance to cellular nucleases and their biological half-life (Cooper et al., 1999; Roth and Yarmush, 1999; Patil et al., 2005).

Screening for active PLCγ1 ASO was performed in the mouse T cell line, EL4-IL-2 using 20 μM of each ASO (ASO 1-4) for electroporation. Antisense sequences tested in this study showed a differential ability to downregulate PLCγ1 protein. This observation is common in antisense screening studies, which report some antisense sequences showing different levels of antisense activity while other sequences were not capable of reducing expression of their target gene at all (Bennett et al., 1994; Dean et al., 1994; Duff et al., 1995; Alahari et al., 1996; Dean et al., 1996; Hill et al., 1999). It has been suggested that proteins involved in RNA synthesis, processing, transport, translation, and degradation may shield certain target mRNA sites or affect the hybridization affinity of some oligonucleotides, thus affecting antisense efficacy

in an unpredictable fashion (Cooper et al., 1999; Freier et al., 2001). Our data showed that ASO3, designed using design approach B, demonstrated the highest level of antisense activity when compared with the other 3 antisense sequences. ASO3 markedly reduced PLCγ1 protein level by 40% and was thus chosen for further *in vitro* profiling.

In this present study, one out of the two antisense sequences designed by approach B was found to be effective in inhibiting PLCγ1 protein expression whereas none of the two sequences designed by approach A was found to be active. This observation seems to support earlier reports on the use of Sfold and Oligo Walk to improve the "hit rate" for active ASO discovery (Ding and Lawrence, 2001; Matveeva et al., 2003). However, more antisense sequences ought to be designed and screened to confirm this. Further, active ASO3 contains a TCCC motif, which was shown by Tu et al. (1998) to be associated with antisense activity. Taken together, the present findings implicate that the use of Sfold and Oligo Walk to predict probable effective antisense sequences and the inclusion of TCCC motif in the antisense sequence greatly improve the chances of identifying effective ASOs.

4.3. ASO3 reduces PLCγ1 mRNA expression by an RNase H-mediated mechanism

ASOs have been described as exogenous regulators of gene expression that exert their effects either by promoting RNase H-mediated mRNA degradation or by steric hindrance, blocking translation of target protein (Crooke et al., 1998). The antisense mechanism of ASO3 in this study was investigated by monitoring PLCγ1 mRNA level in PLCγ1 ASO-treated EL4.IL-2 cells. ASO3 was found to significantly reduce

PLCγ1 mRNA level by 40%, demonstrating that it acts by an RNase H-dependent mechanism where it activates RNase H to cleave PLCγ1 mRNA in the mRNA/ASO duplex, resulting in destruction of PLCγ1 mRNA and therefore, inhibition of translation of PLCγ1 protein.

4.4. ASO3 decreases PLCγ1 mRNA in a concentration-dependent manner

Our findings showed that cells treated with increasing concentrations of ASO3 (10 – 30 μM) exhibited concentration-dependent inhibition of PLCγ1 mRNA. ASO3-mediated reduction reached a plateau above 20 μM of electroporated ASO3, indicating that 20 μM is the minimum concentration of ASO3 required to achieve maximum inhibition of PLCγ1 mRNA. In contrast, scrambled control oligonucleotide (10 – 30 μM), that has the same base composition as ASO3 but in a reversed order, failed to alter PLCγ1 mRNA level in EL4.IL-2 cells, thus demonstrating the specific action of ASO3.

4.5. ASO3 maximally reduces PLCγ1 mRNA at 6 h and PLCγ1 protein at 24 h posttransfection

In this present study, we examined the profile of ASO3-mediated PLCγ1 mRNA downregulation in EL4-IL-2 cells at 6 h intervals for 24 h. Our findings showed that PLCγ1 mRNA was maximally downregulated at 6 h after electroporation and it returned to almost the same level as the controls at 24 h after ASO3 transfection. We also investigated the profile of PLCγ1 protein inhibition by ASO3 for two days at 12 h intervals. PLCγ1 protein was found to be maximally inhibited at 24 h posttransfection and it returned to almost the same level as the controls at 48 h after electroporation. Our data showed that maximal PLCγ1 mRNA depletion preceded several hours before

maximal reduction in PLCγ1 protein, suggesting that PLCγ1 protein may have a long half-life, thus requiring several hours to substantially lower its protein level.

4.6. ASO3 selectively inhibits PLCγ1, but not PLCγ2 expression in a sequence-specific manner

Our present data showed that ASO3-treated cells exhibited significant reduction in PLCγ1 expression while scrambled control oligonucleotide, which has the same base composition as ASO3 but in a reversed order, failed to demonstrate any change in PLCγ1 expression in EL4.IL-2 cells. In addition, we found that ASO3 has no effect on PLCγ2 mRNA level, indicating that ASO3 does not cross-react with PLCγ2 mRNA. Taken together, these data positively demonstrate that ASO3 is highly selective for PLCγ1 mRNA, significantly decreasing its expression in a sequence-specific manner.

The specificity of antisense action observed in our study reflects the attractive value of antisense technology being increasingly used as an alternative to small molecule inhibitors for research and therapeutic purposes as antisense inhibition of gene expression can be achieved more specifically and is thus less likely to be associated with undesirable non-target specific side effects.

4.7. ASO3 reduces anti-CD3/CD28-induced IP_3 generation and intracellular calcium increase

It is known that upon TCR-CD28 costimulation, PLCγ1 becomes activated and hydrolyzes PIP_2 into two intracellular second messengers, IP_3 and DAG (Weiss et al., 1991). The former product causes an increase in cytosolic Ca^{2+} by releasing Ca^{2+} stored in the endoplasmic reticulum and by allowing the entry of extracellular Ca^{2+}

through CRAC channel while the latter activates PKC and Ras-GRP (June et al., 1990; Scharenberg and Kinet, 1998; Kazanietz et al., 2000). All these downstream targets of PLCγ1 activation play important roles in T cell activation (Marais et al., 1993; Crabtree and Clipstone, 1994; Marais et al., 1998). Therefore, in our present study, we investigated the effects of ASO3 on two of the downstream targets of PLCγ1 activation, IP_3 production and intracellular Ca^{2+} mobilization. Our data demonstrated that ASO3-mediated PLCγ1 protein downregulation resulted in significant reduction in IP_3 levels and intracellular Ca^{2+} increase in EL4.IL-2 cells stimulated by anti-CD3/CD28. Consistent with our findings is a study by Kang and colleagues (2003) who reported that inhibition of Itk and PLCγ1 activation by Rosmarinic acid inhibited generation of IP_3 and Ca^{2+} mobilization as well as IL-2 expression and subsequent T cell proliferation. Hence, ASO3 characterized in our study was expected to affect IL-2 production and T cell proliferation.

4.8. ASO3 decreases TCR-CD28 costimulation-induced IL-2 production

IL-2 expression is a hallmark of T-cell activation (Jan and Kaminski 2001). The IL-2 gene is under the regulation of three transcription factors namely, AP-1, NFκB and NFAT and full activation of IL-2 gene transcription requires that all the three transcription factors bind to the promoter region of the gene (Rothenberg and Ward, 1996; Lindholm et al., 1999). These transcription factors are in turn, regulated by MAPK pathway, calcineurin and PKC which are under the control of targets downstream of PLCγ1 activation (Jain et al., 1993; Marais et al., 1993; Crabtree and Clipstone, 1994; Ueda Y et al., 1996; Marais et al., 1998). Therefore, in this study, we examined the effect of PLCγ1 ASO on anti-CD3/CD28 induced IL-2 production in EL4.IL-2 cells. Our present data showed that antisense inhibition of PLCγ1 protein

with increasing concentrations of ASO3 (10 to 30 μM) resulted in a concentration-dependent reduction in IL-2 level in anti-CD3/CD28-stimulated EL4.IL-2 cells. This is consistent with the concentration-dependent downregulation of PLCγ1 mRNA demonstrated in our study, indicating that the observed IL-2 inhibition is a consequence of antisense inhibition of PLCγ1 gene expression by ASO3. On the other hand, scrambled control oligonucleotide did not exhibit any inhibitory effect on IL-2 production. These findings are in line with earlier studies demonstrating impaired IL-2 expression and TCR activation in PLCγ1-deficient cell lines (Irvin et al., 2000; Dienz et al., 2003). Hence, PLCγ1 indeed plays an important role in IL-2 production in T cells.

Our data on PLCγ1 gene inhibition and subsequent inhibition of IL-2 production by PLCγ1 ASO demonstrated the advantages of using antisense technology for the study of gene function as inhibition of gene expression with antisense oligonucleotides can be achieved more rapidly and at a relatively lower cost compared with generating knockout mice for functional genomics.

4.9. ASO3 and scrambled control oligonucleotide reduce anti-CD3/CD28 stimulation-induced T cell proliferation

Upon activation, T cells produce IL-2 and the secreted IL-2 binds to the IL-2R on T cell surface, promoting proliferation in an autocrine manner (Rooney et al., 1995). Hence, in this present study, we investigated the effect of ASO3 on T cell proliferation. Our data showed that ASO3 and unexpectedly, scrambled control oligonucleotide significantly reduced proliferation of anti-CD3/CD28-simulated EL4.IL-2 cells. This observation could be explained with several possible reasons.

Firstly, the incorporation of PS moieties into oligonucleotides have been shown in previous studies to have resulted in these oligonucleotides having greater affinity for proteins, leading in many cases to nonspecific effects (Stein et al., 1996). A study by Cazenave et al. (1989) showed that PS oligonucleotides in both frog oocyte and wheatgerm extract *in vitro* translation systems, bind to enzymes involved in protein synthesis and nonspecifically inhibited protein synthesis. In addition, Shaw and colleagues (1997) reported that PS oligonucleotides nonspecifically bind to clotting factors such as thrombin and impaired clotting. Further, other proteins such as serum albumin, human immunodeficiency virus type I (HIV-1) reverse transcriptase, HIV gp120 and DNA polymerase-α and -β have also been shown to interact nonspecifically with and be inhibited by PS oligonucleotides (Gao et al., 1992; Maury et al., 1992; Stein et al., 1993). In this present study, the observed reduction in proliferation in scrambled control oligonucleotide-treated cells could be a result of the PS oligonucleotides binding nonspecifically to molecules important for T cell proliferation. This nonantisense effect could have masked the antisense effect of ASO3 on EL4.IL-2 proliferation and could be overcome with the use of chimeric oligonucleotides consisting of a stretch of five to seven PS-modified bases in the middle of the molecule and 2′-O-modified bases at the 5′- and 3′-ends of the oligonucleotide. Chimeric oligonucleotides have reduced PS content and are thus likely to be associated with less nonspecific effects than full backbone PS-modified oligonucleotides. However, our observed data could also be due to PS oligonucleotides interfering nonspecifically with MTS assay components, thus resulting in low absorbance readings obtained with ASO3- and scrambled control oligonucleotide - treated cells. This problem could be overcome by using an alternative proliferation assay such as [^{3}H]thymidine incorporation assay which uses

[^{3}H]thymidine instead of MTS tetrazolium compound and PES used in MTS assay to monitor cell proliferation.

It has also been previously reported that several motifs in oligonucleotides are associated with nonantisense effects. Oligonucleotides that are G-rich are well known for their nonantisense effects (Ecker et al., 1993; Bennett et al., 1994), which have been attributed to the tendency of these oligonucleotides to form G-quartet structures that then interfere with biological processes (Wyatt and Stein, 1999). In addition, an earlier study by Bergan et al. (1994) showed that an oligonucleotide containing GGC motif exhibited nonantisense activity by inhibiting tyrosine kinase p210$^{bcr\text{-}abl}$ activity without affecting its protein level. Our present findings on reduced cell proliferation in ASO3-treated EL4.IL-2 cells could be a truly antisense effect mediated by PLCγ1 ASO while the observed reduction in cell proliferation in scrambled control oligonucleotide-treated EL4.IL2 cells could be due to a nonantisense effect caused by a particular unidentified "bad" motif present only in the scrambled control oligonucleotide. Hence, further studies involving the inclusion of additional controls with different sequences such as sense and mismatch controls are needed to verify this.

In this present study, our findings on selective inhibition of PLCγ1, but not PLCγ2 expression by ASO3 and subsequent reduction of anti-CD3/CD28 induced IP_3 generation, intracellular calcium increase and IL-2 production by ASO3 but not by scrambled control implicate that the reduction in proliferation observed in ASO3-treated cells is likely a consequence of PLCγ1 protein downregulation mediated by ASO3. However, further investigations involving the use of oligonucleotides

associated with less nonspecific effects such as chimeric oligonucleotides and additional controls such as sense and mismatch controls are needed to confirm the antisense effect of ASO3 on T cell proliferation.

5. CONCLUSION

PLCγ1 plays a pivotal role in the activation of T cells. This is the first study to report inhibition of TCR-CD28 costimulation-induced IL-2 production in EL4.IL-2, a mouse T cell line by an ASO targeting mouse PLCγ1 mRNA.

The limited gene-walk in this study identified an active PLCγ1 ASO, ASO3 that markedly inhibited PLCγ1 protein expression. ASO3-mediated PLCγ1 inhibition was further demonstrated at the mRNA level in a concentration-dependent manner while no effect was observed in scrambled control-treated cells or PLCγ2 expression. Time course study on ASO3-mediated PLCγ1 gene inhibition profile revealed maximum mRNA downregulation at 6 h after electroporation and maximum protein reduction at 24 h after transfection. Subsequent measurement of IP_3, intracellular calcium and IL-2 levels in PLCγ1 ASO-treated murine EL4.IL-2 cells showed significant reduction in all the three parameters. Further, our study demonstrated that ASO3-treated cells and surprisingly, scrambled control-treated cells exhibited significant reduction in cell proliferation following TCR-CD28 costimulation. It is likely that the reduction in proliferation observed in ASO3-treated cells is a consequence of PLCγ1 protein downregulation mediated by ASO3, however, further investigations involving the use of oligonucleotides associated with less nonspecific effects such as chimeric oligonucleotides and additional controls such as sense and mismatch controls are needed to confirm the effect of ASO3 on T cell proliferation. Our present work discovered and characterized an ASO capable of specifically downregulating PLCγ1 expression and subsequently inhibiting TCR/CD28 stimulation-induced IL-2 production in the mouse T cell line, EL4.IL-2. If inhibitors of key molecules involved in T cell activation, such as PI3K inhibitors (wortmannin and LY-294002), and PLCγ inhibitor (U-73122), which inhibit all isoforms of PI3K and PLCγ respectively, were

used in this study, it is likely that IL-2 production would also be inhibited. However, these inhibitors are more likely to be associated with undesirable non-specific effects compared with PLCγ1 ASO which specifically inhibits the γ1, but not γ2 isoform. More extensive gene-walking in future study may identify an even more potent PLCγ1 ASO capable of inhibiting mouse PLCγ1 expression and T cell activation to a greater extent. Future studies employing multiple ASOs or small interfering RNAs (siRNAs) targeting specifically at key molecules involved in T cell activation ─ PLCγ1 and perhaps PI3K, may discover promising therapeutics that could completely attenuate T cell activation.

Taken together, the present findings implicate that antisense inhibition of PLCγ1 may have therapeutic potential for the treatment of T cell-dependent disorders. Our data in this present study exemplify the attractive value of antisense technology as an alternative to small molecule inhibitors and knockout mice for research purposes as antisense inhibition of gene expression can be achieved more specifically than with small molecule inhibitors and more rapidly and at a relatively lower cost compared with generating knockout mice for gene function studies.

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7. APPENDIX

List of Reagents and Solutions

Lysis buffer

Components	Stock concentration	Final concentration	Prepare 500 µl
			Volume (µl)
Tris-HCl (pH 7.5)	1000 mM	50 mM	25
NaCl	2000 mM	150 mM	37.5
Triton X-100	100%	1%	5
Na_3VO_4	200 mM	2 mM	5
NaF	1000 mM	34 mM	17
PMSF	200 mM	5 mM	12.5
Aprotinin	5000 µg/ml	10 µg/ml	1
Leupeptin	5000 µg/ml	10 µg/ml	1
SDS	10%	0.1%	5
Autoclaved Milli-Q water	-	-	391

5X sample buffer (store at –20 °C)

Components	Stock concentration	Final concentration	Prepare 50 ml
			Amount
Tris-HCl (pH 6.8)	3 M	0.313 M	5.2 ml
Glycerol	100%	50%	25 ml
2-mercaptoethanol	100%	25%	12.5 ml
SDS	-	10%	5 g
Bromophenol blue	1%	0.0625%	3.1 ml
Autoclaved Milli-Q water	-	-	4.2 ml

10% separating gel

Components	Stock concentration	Final concentration	Prepare 15 ml
			Volume (µl)
Tris-HCl (pH 8.8)	1.5 M	0.373 M	3730
Acrylamide/Bis solution (37.5:1)	30%	10%	4930
SDS	10%	0.1%	150
Glycerol	10%	0.1%	150
APS	10%	0.05%	77
TEMED	100%	0.05%	7.3
Autoclaved Milli-Q water	-	-	5956

Stacking gel

Components	Stock concentration	Final concentration	Prepare 5 ml
			Volume (µl)
Tris-HCl (pH 6.8)	0.5 M	0.125 M	1250
Acrylamide/Bis solution (37.5:1)	30%	3.75%	625
SDS	10%	0.1%	50
Glycerol	10%	0.1%	50
APS	10%	0.03%	15
TEMED	100%	0.1%	5
Autoclaved Milli-Q water	-	-	3005

10X electrophoresis buffer

Components	Final concentration	Prepare 2 L
		Amount
Tris	0.25 M	60.57 g
Glycine	1.92 M	288.27 g
SDS	1%	20 g
Milli-Q water	-	q.s

10X transfer buffer (store at 4°C)

Components	Final concentration	Prepare 1 L
		Amount
Tris	1 M	121.14 g
Glycine	1.92 M	144.14 g
Milli-Q water		q.s.

10X Tris-buffered saline (TBS)

Components	Final concentration	Prepare 1 L
		Amount
Tris-HCl (pH 7.5)	1 M	121.14 g
NaCl	9%	90 g
Milli-Q water	-	q.s.

Tween 20-Tris- buffered saline (TTBS)

Components	Stock concentration	Final concentration	Prepare 2 L
			Volume (ml)
TBS	10X	1X	200
Tween 20	100%	0.05%	1
Milli-Q water	-	-	1799

DEPC-treated Milli-Q water

Components	Stock concentration	Final concentration	Prepare 2 L
			Volume (µl)
DEPC	100%	0.01%	200
Milli-Q water	-	-	1800

DEPC was added to Milli-Q water and stirred for 1 h. The solution was allowed to stand overnight and then autoclaved.

10X PBS pH 7.3

Components	Final concentration (M)	Prepare 1 L
		Amount
NaCl	1.37	80 g
Na_2HPO_4	0.027	11.5 g
$KH_2PO_4.H_2O$	0.043	2 g
KCl	0.014	2 g
Milli-Q water	-	q.s.

Coating buffer (0.1 M carbonate, pH 9.5)

Components	Prepare 1 L
	Amount
$NaHCO_3$	8.4 g
Na_2CO_3	3.56 g
Milli-Q water	q.s.