MECHANISMS AND CONSEQUENCES OF THY-1 SHEDDING

by

JOHN EDWIN BRADLEY

JAMES S. HAGOOD, COMMITTEE CHAIR
PETER J. DETLOFF
JOANNE E. MURPHY-ULLRICH
THOMAS M. RYAN
TIM M. TOWNES

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JOHN EDWIN BRADLEY

BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Idiopathic Pulmonary Fibrosis (IPF) is characterized by chronic fibrosis in the lung interstitium of unknown etiology, and causes death within 2 to 4 years after diagnosis. There are no clinical interventions save for lung transplantation that give any survival benefit. Aberrant collagen deposition and aggregates of proliferating fibroblasts and myofibroblasts called fibroblastic foci (FF) are the classic features of IPF. Therefore, the cell type most often implicated as pathogenic in IPF is the fibroblast, especially its differentiated phenotype, the myofibroblast. Fibroblasts are a remarkably heterogeneous cell type. Expression of the cell surface glycoprotein Thy-1 (Thymocyte differentiation antigen 1) delineates a normal phenotype from a more fibrotic one. Thus, mechanisms that regulate Thy-1 expression could perhaps be exploited to abrogate phenotypes associated with disease. The initial goals of my work were to elucidate one such mechanism, namely Thy-1 shedding. To accomplish this, four specific aims were devised. They were to (1) determine the mechanism(s) by which pulmonary fibroblasts shed Thy-1 from their cell surface in response to pro-fibrotic stimuli. In conjunction with this, (2) characterize the C-terminus of Thy-1 released from fibroblasts and (3) identify residues of Thy-1 that modulate susceptibility to or are required for its shedding. Finally, (4) measure the concentrations of soluble Thy-1 and of identified sheddases in the bronchoalveolar lavage (BAL) fluid of patients with lung fibrosis and determine the degree to which they correlate. During the course of pursuing these aims it was
determined that all the monoclonal antibodies to human THY1 available in the lab were incapable of recognizing delipidated THY1, e.g. soluble THY1. Taking this into consideration, we reevaluated the solubility of THY1 we reported detecting in the conditioned media (CM) of normal human lung fibroblast (NHLF) and found it to be entirely insoluble. From our and other’s reporting there is a need to develop antibodies to THY1 that take into consideration the conformation of delipidated THY1. Additionally, the conformation of recombinant forms of THY1 used as controls in assays that rely on antibody recognition must be evaluated carefully. Beyond antibody recognition, the conformation of recombinant forms of THY1 used to elucidate its function should also be evaluated.

Keywords: THY1, GPI Anchor, Fibroblast, Antibody Recognition, IPF
DEDICATION

To my grandmother, Iris Goring, whose personal sacrifices through life gave me the chance to continue my education.

To my parents, Johnny and Shirley Bradley, for your love and support throughout my life.

To Alana Czernobil, stage name, for loving me the most. I love you the “most-est”.
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<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACEI</td>
<td>Angiotensin Converting Enzyme I</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>CD90</td>
<td>Cluster of Differentiation 90</td>
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<tr>
<td>CE</td>
<td>Cell Extract</td>
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<tr>
<td>CM</td>
<td>Conditioned Media</td>
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<tr>
<td>CpG</td>
<td>cytosine-guanine</td>
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<td>CSF</td>
<td>cerebral spinal fluid</td>
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<tr>
<td>DAF</td>
<td>Decay Accelerating Factor</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>FF</td>
<td>fibroblastic foci</td>
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<tr>
<td>FR</td>
<td>Folate Receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycophosphatidylinositol</td>
</tr>
<tr>
<td>GPLD1</td>
<td>GPI-specific phospholipase D1</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic Pulmonary Fibrosis</td>
</tr>
<tr>
<td>MV</td>
<td>membrane vesicle</td>
</tr>
<tr>
<td>NHLF</td>
<td>Normal Human Lung Fibroblast</td>
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<tr>
<td>NR</td>
<td>Non-reducing</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PI-PLC</td>
<td>Phosphatidylinositol-Specific Phospholipase C</td>
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<tr>
<td>SFM</td>
<td>Serum Free Media</td>
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<tr>
<td>SLB</td>
<td>surgical lung biopsy</td>
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<tr>
<td>Thy-1</td>
<td>Thymocyte differentiation antigen 1</td>
</tr>
<tr>
<td>TRAIL-R3</td>
<td>TNF-related Apoptosis-inducing Ligand Receptor 3</td>
</tr>
<tr>
<td>VSG</td>
<td>Variant Surface Glycoprotein</td>
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<td>WT</td>
<td>Wild Type</td>
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CHAPTER 1

INTRODUCTION

Idiopathic Pulmonary Fibrobrrosis

IPF is the most common of the Idiopathic Interstitial Pneumonias within the diseases known as Diffuse Parenchymal Lung Diseases, and has the worst prognosis [1], [2]. Across the United States, there are an estimated 40,000 to 130,000 IPF patients [1]. In the majority of IPF cases, the onset is later in life, after 50 years of age. Mean survival for an IPF patient is 2 to 4 years after diagnosis [3]. IPF is somewhat more common in males, though there is no clear predominance in any race, ethnicity, or geographical location [1], [3]. As the name implies, the two major features of IPF are an unknown etiology and chronic fibrosis in the lung interstitium, which results from excessive and abnormal extracellular matrix (ECM) deposition [1–3]. IPF is also associated with diffuse interstitial inflammation, although the contribution of inflammation to onset and progression of IPF is controversial [1–3]. The inflammation and aberrant ECM deposition, mostly collagen, cause alveolar walls to thicken, the consequence being an increased barrier to gas diffusion across the air/blood interface [1], [2]. As one would expect, patients suffer from severe dyspnea in addition to a nonproductive cough. With increasing fibrosis comes deteriorating pulmonary function. Specifically, the lungs’ vital capacity, compliance, and volume undergo a progressive reduction during the course of the disease [3].
Unfortunately, the aforementioned IPF signs and symptoms are nonspecific, and also associated with a plethora of other pulmonary disorders. However, the prognosis for someone with IPF versus someone with another pulmonary disorder can be radically different [2]. This demonstrates the need for clinical biomarkers that allow an early and accurate diagnosis of IPF, in order to start the patient on a correct therapeutic regimen. Despite the inherent risks and expanded use of high resolution computer tomography scans for IPF diagnosis, surgical lung biopsy (SLB) affords the best likelihood of an accurate diagnosis and the pathological features observed in histological slides provide insights into the etiology of IPF. The prominent histopathologic features are “swirling” aggregates of proliferating fibroblasts and myofibroblasts called FF, thickened alveolar walls, and massive collagen deposits. Other features include mild to moderate infiltration by lymphocytes, a likely source of the inflammation, and abnormal alveolar epithelium epitomized by hyperplasia of type II pneumocytes [2]. The presence and number of FF on biopsy are correlated with poor prognosis [4].

IPF is associated with abnormal activity in both epithelial and mesenchymal cell types. The association of hyperplastic Type II pneumocytes which are unable to restore alveolar epithelium with pervasive fibroblasts and myofibroblasts has led many in the field to believe IPF is a result of aberrant signaling in the wound repair process [1]. Despite increased understanding of pathophysiologic abnormalities in IPF, there is still a lack of effective therapy or reliable and definitive diagnostic techniques. Currently, the only recommended pharmaceuticals are those meant to combat inflammation, though they provide no survival benefit to the patient. The only known intervention to provide such a benefit is a lung transplant [2]. Thus, to develop more effective therapies and
better diagnostic techniques, further research into the mechanisms that underlie fibroblast activation as well as the role epithelial and mesenchymal cross-talk has to play in fibrosis is required. Additionally, clinical biomarkers of disease activity are needed to assess response to new therapy.

Fibroblast and Myofibroblast

The cell type most often implicated in the pathogenesis of IPF is the fibroblast and its differentiated phenotype, the myofibroblast. As mentioned earlier, these cell types represent the primary cellular component of FF, the number of which correlates with a negative prognosis in IPF patients [1–3]. Fibroblasts are the principal producers of collagen, the main component of abnormal ECM deposition observed in IPF [1]. Specifically, fibroblasts produce collagen types I, III, and IV, and once differentiated into myofibroblasts produce greater amounts of all three [5]. In a normal wound healing scenario, myofibroblasts produce collagen to reestablish the ECM of injured tissue providing the scaffold upon which cells re-populate the damaged area. In the lung, hyperplastic type II pneumocytes are thought to subsequently differentiate into type I cells, which restore the normal alveolar surface. Myofibroblasts share certain characteristics with smooth muscle cells. Specifically, myofibroblasts have contractile elements composed of α smooth muscle actin, which is utilized to distinguish them from normal fibroblasts. Myofibroblasts contract damaged tissue, reducing the empty regions that occur from cell loss [6–8]. The persistence or excessive abundance of myofibroblasts prevents resolution of normal wound healing and leads to fibrosis. This is evident by their
persistence within FF. Myofibroblasts are also resistant to apoptosis [8]. Given that fibroblasts and myofibroblasts are the principal cellular component of FF, and that myofibroblasts appear to be the main source of pathological ECM in IPF, investigating factors that mediate their differentiation and activity would have obvious potential for developing clinical interventions.

Undifferentiated fibroblasts do not consist of a single phenotype. Fibroblasts from different tissues can have vastly differing phenotypes. Even within a particular tissue, fibroblasts are heterogeneous. Surface expression of Thy-1 has been used to characterize fibroblast subpopulations in a number of tissues, including myometrium, orbit, zone II tendon, synovial fluid, skin, lung, and liver [9–13]. For pulmonary fibroblasts, differential expression of Thy-1 delineates polar phenotypes with respect to potential for differentiation into myofibroblasts, response to and production of profibrotic cytokines, and localization in areas of active fibrosis [13]. Specifically, rat Thy-1 (−) pulmonary fibroblasts at baseline as well as in response to fibrogenic mediators have greater myofibroblastic differentiation as evaluated by enhanced contractility and myogenic gene expression of MyoD, myocardin, myf5, and myogenin [14]. TNF-α, IL-1β, and PDGF-AA all elicit a unique or greater fibrotic response in Thy-1 (−) fibroblasts, in particular with regard to activation of latent TGF-β [13]. Within the aforementioned FF the myofibroblasts are predominantly Thy-1 (−), whereas the majority of normal lung fibroblasts express Thy-1 [4]. Thy-1 deficient mice are viable and have normal life expectancy. They have abnormalities of pulmonary septation resulting in hypoalveolar lungs in the first two weeks of life, however these differences are not statistically significant in adult mice. However, intratracheally administered bleomycin induces a
more severe lung fibrosis in Thy-1 deficient mice than it does in WT litter mates as indicated by a greater accumulation of myofibroblasts, collagen, and increased activation of TGF-β [15]. Therefore, mechanisms that regulate Thy-1 expression may consequently regulate the pulmonary fibroblast phenotypes. With an understanding of such mechanisms, pharmaceuticals developed to alter Thy-1 expression have the potential to abrogate phenotypes associated with disease.

Membrane Bound Thy-1

Thy-1 or Cluster of Differentiation 90 (CD90) was initially discovered in an attempt to raise antiserum against leukemia-specific antigens from C3H in AKR and vice versa [16]. Currently, Thy-1 is recognized as a member of the immunoglobulin superfamily and is evolutionarily conserved; significant homology exists among multiple species including squid, chicken, frogs, mice, rats, and humans [17]. In addition to fibroblasts, Thy-1 is expressed in neurons, retinal ganglion cells, activated endothelial cells, mesangial cells, and hematopoietic and mesenchymal stem cells [13], [18]. The murine thy1 locus is mapped to chromosome 9, at which two alleles encode the proteins designated Thy1.1 and Thy1.2. Both are 162 amino acids (AA) in length but differ by a single AA at position 89. Thy1.1 has an arginine while Thy1.2 has a glutamine at this position. In humans, THY1 is mapped to chromosome 11q22.3, at which one allele encodes a 161 AA protein [18]. Both murine and human Thy-1 are initially expressed in proform then undergo several post translational modifications. The first 19 AAs of Thy-1 act as a signal peptide that targets it into the ER and is later removed [19]. Thy-1 is also
N-glycosylated at two [20] to three sites [21] that account for nearly 30% of its mass [18], which ranges from 25 to 37kDa [21]. Between different tissues, the carbohydrate moiety composition may vary dramatically [22], [23].

Immature murine Thy1 and human THY1 are initially embedded in the inner leaflet of the ER by residues downstream of CYS 131 and 130, respectively. This C-terminal transmembrane domain is cleaved away and replaced by a glycophosphatidylinositol (GPI) anchor at the C-terminal CYS. The GPI anchor is composed of two fatty-acyl moieties that tether Thy-1 to the cell surface [20]. In addition, the GPI anchor participates in targeting Thy-1 to a specific membrane micro domain. Localization of Thy-1 to its native micro domain is required for its signaling [24], [25] but is also thought to afford endothelial Thy-1 protection from GPI specific lipases in serum [26]. Decay Accelerating Factor (DAF) and TNF-related Apoptosis-inducing Ligand Receptor 3 (TRAIL-R3) are both GPI-anchored proteins and targeted to unique specific membrane micro domains. Immature DAF and TRAIL-R3 undergo a similar pros-translational modification to Thy-1 in that both contain a region that initially embeds into the inner leaflet of the ER but is later cleaved away and replaced with a GPI anchor. Experiments with eGFP fused to the C-terminus of DAF or TRAIL-R3 demonstrated that once the trans-membrane domain was replaced with a GPI anchor, eGFP processed the same distinctive partitioning profile in fractions taken from a density gradient centrifugation in Triton X-100 of the proteins it was fused to [27].
Soluble Thy-1

Thy-1 does not exist solely in a membrane bound form. Soluble forms of THY1 have been reported in serum, cerebral spinal fluid (CSF), wound fluid from venous leg ulcers, and synovial fluid from joints in rheumatoid arthritis [21], [28]. Speculated methods by which cells produce soluble Thy-1 include an alternative mRNA splice variant omitting addition of the GPI anchor, or an enzyme that cleaves Thy-1 away from the cell surface. Interestingly, the glycosylation pattern of soluble Thy-1 can differ from that of presumptive sources. For example, soluble THY1 detected in CSF has slightly higher mass than THY1 in cerebral cortex membranes. This differential size is attributed to unique glycosylation patterns; specifically, soluble THY1 is resistant to Endo H, which indicates low mannose content, whereas Thy-1 in cerebral cortex membranes is Endo H susceptible. However, both the soluble and membranous forms are identical in size when all the N-glycosyl groups are removed [21].

Soluble THY1 in CSF could possibly originate from a region of the brain other than the cerebral cortex. In rats, the carbohydrate moiety of brain Thy1 has considerably less sialic acid content than that of thymocytes [28], though variation between regions of the brain is not well characterized. These findings suggest that the GPI anchor is severed in close proximity to the protein moiety, for example, within the GPI anchor itself or at a protease target just up stream. GPI-specific phospholipase D1 (GPLD1) [29–31], Angiotenin Converting Enzyme I [32–34], and Phosphatidylinositol-Specific Phospholipase C (PI-PLC) [26] cleave within the Thy-1 GPI anchor. The former two phospholipases are produced in mammals, whereas the latter is of bacterial origin. These enzymes have distinct enzymatic requirements in order to cleave Thy-1 from the cell
surface. Detergents are needed by exogenous GPLD1 and Angiotensin Converting Enzyme I (ACEI) to cleave Thy-1, whereas PI-PLC does not have such a requirement [26], [33]. Though, detergents are not required by PI-PLC, the susceptibility of Thy-1 to cleavage varies form one cell type to another [35]. This is all relevant given that serum contains GPLD1 and as a consequence Thy-1 positive endothelial cells as well as circulating T-cells are susceptible to its action, yet the concentration of serum THY1 is only 105 to 251ng/mL [28]. Localization of Thy-1 to cholesterol-rich lipid rafts is thought to protect it from GPLD1 present in serum [26]. The exact mechanism(s) of Thy-1 shedding and possible roles of shedding in normal biology and in disease have yet to be determined.

Regardless of the mechanism, shedding of Thy-1 could very likely play an important role in facilitating its complete removal from the cell surface, given its unusually slow turnover rate [36]. Cultured lung fibroblasts shed Thy-1 into the media when treated with various profibrotic cytokines, such as IL-1β and TNF-α [15], yet the soluble and membrane bound THY1 have indistinguishable migration speeds through an acrylamide gel. As of yet, it is unknown whether Thy-1 in itself has a role in its own regulation. The biological functions of soluble Thy-1 remain unclear. But, it is important to note that Thy-1 lacking a complete GPI anchor very often becomes unrecognizable by antibodies against its membrane bound form [37]. Because of this, a number of caveats arise for detecting soluble THY1 in bodily fluids by methods that employ such antibodies. Is THY1 lacking a complete GPI anchor in bodily fluids going undetected? Moreover, antibodies demonstrated to be incapable of recognizing THY1 without a complete GPI anchor but detect THY1 in aqueous bodily fluids raise the question
whether the detected THY1 is truly soluble, e.g. delipidated. THY1 identified as a disease biomarker would mean measuring the absolute THY1 concentration in a single patient would be needed. To do so, the antibodies must be capable of recognizing delipidated THY1 and have a similar affinity to the source of THY1, e.g. recombinant, used to establish a standard curve and the THY1 being measured. To clarify these issues, an assay is required to establish whether already established and newly developed antibodies to THY1 have a predilection for complete or delipidated THY1. Specifically, the monoclonal antibodies from clones AS02 and 5E10 are purported to be capable of detecting soluble THY1 though each raised against membrane bound THY1 [28], [38], [39].

Shedding of Thy-1 could potentially elicit a number of effects. Fewer Thy-1 molecules at the cell surface could directly affect the cell by limiting ligand binding and signal transduction. Soluble Thy-1 could also retain a comparable affinity for ligands of its membrane bound form, making ligand unavailable to Thy-1 at the surface of the cell from which it was shed as well as on neighboring cells. Conversely, conformational change induced by removal of the GPI anchor may allow soluble Thy-1 to garner new ligands. In either case, soluble Thy-1 would signal distal to the cell that shed it.

Summary

Despite decades of excellent research on IPF, the cause remains unknown and the prognosis remains dire. Mean survival for an IPF patient is 2 to 4 years after diagnosis [3]. During those few years, IPF causes patients a great deal of pain and discomfort.
Many deaths stem from complications that arise during sudden acute exacerbations of the disease. Recommended pharmaceutical therapies do not afford any survival benefit. Today, lung transplant remains the only clinical intervention to provide survival benefit to IPF patients. Further research into basic mechanisms in IPF could translate into a plethora of clinical applications. Earlier and accurate diagnosis could reveal a window of time that allows clinical intervention to have an impact. In addition, a less invasive diagnostic technique than SLB would eliminate the inherent risks involved. The unknown etiology and often nonspecific, delayed clinical presentation makes choosing a target early in the disease course, such as a specific metabolic or signaling pathway, mutation, or pathogen, difficult to investigate in IPF. However, the fibroblast and its differentiated phenotype the myofibroblast are an attractive “final common pathway” in fibrosis. In fibroblasts, Thy-1 cell surface expression both modulates and delineates divergent phenotypes that associate with disease and non-disease states. Specifically, the phenotype of Thy-1 (−) fibroblasts promotes fibrogenesis. Thus, the means by which fibroblast regulate Thy-1 could be utilized in combating IPF.

My first specific aim was to determine the mechanism(s) by which pulmonary fibroblasts shed Thy-1 from their cell surface in response to pro-fibrotic stimuli. Release of Thy-1 could elicit a cascade of effects that may not be limited to fibroblasts. Targeting the mechanism of shedding, as well as elucidating any signaling potential soluble Thy-1 has will require knowing the exact molecular species released. With this in mind, my second specific aim was to characterize the C-terminus of Thy-1 cleaved from fibroblasts treated with pro-fibrotic stimuli or by increasing endogenous levels of “sheddases”. The
latter was also intended to confirm “sheddases” indentified from the experiments in specific aim one.

There existed the potential that none of the candidate “sheddases” in specific aim would be correct. This possibility was addressed in my third specific aim. Should no “sheddases” be identified by experiments for specific aim one, then identifying residues of Thy-1 that modulate susceptibility to or are required for its shedding from pulmonary fibroblasts elicited by pro-fibrotic stimuli will provide clues to other candidates. My fourth specific aim was to measure the concentrations of soluble Thy-1 and of identified sheddases in the BAL fluid of patients with lung fibrosis and determine if a correlation exists between them. Moreover, soluble Thy-1 concentrations in BAL fluid might serve as a diagnostic tool or biomarker, and the procedure to procure BAL fluid is far less invasive than SLB. The totality of my proposal was to elucidate exogenous and endogenous mechanisms that shed Thy-1 from pulmonary fibroblasts in context with fibrosis which could be exploited into treating and diagnosing IPF.
ROLES AND REGULATION OF THY-1, A CONTEXT-DEPENDENT MODULATOR OF CELL PHENOTYPE

by

JOHN E. BRADLEY, GUSTAVO RAMIREZ, JAMES S. HAGOOD
Abstract

Thy-1 or CD90 is a GPI-linked glycoprotein expressed on the surface of neurons, thymocytes, subsets of fibroblasts, endothelial cells, mesangial cells and some hematopoietic cells. Thy-1 is evolutionarily conserved, developmentally regulated, and often has dramatic effects on cell phenotype; however, the effects vary between and in some cases within cell types and tissues, and between similar tissues in different species, indicating that the biological role of Thy-1 is context-dependent. Thy-1 exists in soluble form in some body fluids; however, the mechanisms of its shedding are unknown. In addition, Thy-1 expression can be regulated by epigenetic silencing. Because Thy-1 modulates many basic cellular processes and is involved in the pathogenesis of a number of diseases, it is important to better understand its regulation.
1. Introduction

Thy-1, also known as CD90, is a highly conserved but somewhat enigmatic molecule that can exist in membrane-bound and soluble forms. Although it is most often used as a marker of certain cell types or of ‘‘stem-ness,’’ its presence or absence has significant effects on cellular biology, and its dysregulation is associated with malignancy and fibrotic diseases. Previous reviews have focused on its immunologic and nonimmunologic roles, and mechanisms and consequences of Thy-1-associated signaling [13], [18], [40], [41]. Here, we will consider the regulation of Thy-1 in the context of its pathogenic alterations.

Thy-1 was initially discovered in an attempt to raise antiserum against leukemia-specific antigens from the C3H strain of mouse in the AKR strain and vice versa [42]. It was originally designated theta, but later renamed Thy-1 [43]. In addition to thymocytes and T-cells, a number of other cell types are known to express Thy-1, specifically neurons, retinal ganglion cells, subsets of fibroblasts, vascular pericytes, activated endothelial cells, mesangial cells, and hematopoietic and mesenchymal stem cells. There are important species-specific differences in expression. For instance, Thy-1 is expressed on both peripheral T cells and thymocytes of mice, whereas in humans, Thy-1 is absent in the former and developmentally regulated in the latter [44]. Thy-1 modulates the phenotypes of cells implicated in several disease states, including neuronal injury [45–47], pulmonary fibrosis [14], [41], [48], certain cancers [49–51], Graves’ disease, Graves’ ophthalmopathy (GO) [52], and glomerulonephritis [53].
2. Pathogenic alterations in Thy-1

Several observations associate Thy-1 with the resolution of neuronal injury. Thy-1 is either not expressed on [54] or restricted to the somatodendritic membranes of growing rodent neurons [54], yet accounts for 2.5–7.5% of total protein on axon membranes of mature rat neurons [55]. Thy-1 expression in the nervous system is predominantly neuronal, but some human glial cells also express Thy-1, especially at later stages of their differentiation [56]. Neurite outgrowth is inhibited in Thy-1 (−) neurons made to express either human Thy-1 or mouse Thy-1.2 when grown on a monolayer of astrocytes [57]. In similar conditions, antibodies against Thy-1 or soluble Thy-1 allow neurite outgrowth to occur, presumably by blocking the interaction of Thy-1 with a ligand on astrocytes [57]. Injury to the sciatic nerve in young adult rats causes an initial decline of Thy-1 expression followed by an increase on dorsal root ganglion neurons that coincides with recovery of sensory function [17].

However, presence of Thy-1 on neuron membranes is not sufficient in itself to inhibit neurite outgrowth, but requires correct localization of Thy-1 to its native membrane micro domain to exert an inhibitory effect [58]. Taking these all into account, the prevailing thought is that Thy-1 expressed in neurons, when localized to its native membrane microdomain, inhibits neurite outgrowth and its continued expression in mature neurons likely plays a role in stabilizing them and their junctions. The phenotype of Thy-1 deficient mice is remarkably devoid of major abnormalities involving the nervous system. However, thorough examination revealed subtle phenotypes, including inhibition of hippocampal long-term potentiation in the dentate gyrus, failure to transmit
social cues regarding food selection, and an impaired cutaneous immune response [59–61].

Pulmonary fibrosis is characterized by the development in the lung of fibrotic tissue, which is characterized by excessive and abnormal extracellular matrix [1]. The cell type often implicated in pulmonary fibrosis is the fibroblast, specifically its differentiated phenotype, the myofibroblast [1]. Individual fibroblasts can have vastly differing phenotypes, especially when they originate from different tissues [9], [11], [12], [62], [63]. Even within a particular tissue, fibroblasts are still heterogeneous [41]. For pulmonary fibroblasts, differential expression of Thy-1 is a well-characterized paradigm for distinguishing polar phenotypes with respect to potential for differentiation into myofibroblasts, response to profibrotic cytokines, and localization in areas of active fibrosis [41]. Specifically, rat Thy-1 (–) pulmonary fibroblasts at baseline as well as in response to fibrogenic mediators have greater myofibroblastic differentiation as evaluated by myogenic gene expression and enhanced contractility. Furthermore, they are resistant to apoptosis in a contracting collagen matrix [14]. TNF-α, IL-1β, and PDGF-AA all elicit a unique or greater fibrotic response in Thy-1 (–) fibroblasts, in particular with regard to activation of latent TGF-β [41]. The hallmark feature of IPF is aggregates of proliferating fibroblasts and myofibroblasts called FF [1], increased numbers of which are associated with poor prognosis [4]. Within these foci are predominantly Thy-1 (–) myofibroblasts, whereas the majority of normal lung fibroblasts express Thy-1. Intra-tracheal bleomycin induces more severe lung fibrosis in Thy-1 knockout mice as evidenced by greater accumulation of myofibroblasts, collagen and increased activation of TGF-β [15].
relationship of Thy-1 expression to pathogenic alterations in lung fibroblasts in pulmonary fibrosis is depicted in Fig. 1.

Thy-1 is thought to act as a tumor suppressor in several types of cancer, including nasopharyngeal and ovarian cancer [51]. In ovarian cancer, loss of heterozygosity at 11q23.3-q24.3 is associated with poor prognosis [64] and Thy-1 is mapped to this region [19]. Inducing Thy-1 expression in the tumorigenic ovarian cancer cell line, SKOV-3, by either microcell mediated chromosome 11 transfer [64] or a Thy-1 expression inducible system [50] suppressed tumorigenicity. Concurrently, induced Thy-1 expression in SKOV-3 mediates upregulation of thrombospondin-1 and fibronectin. These genes are differentially expressed in tumorigenic and nontumorigenic hybrid clones as well as being associated with cell differentiation and angiogenesis inhibition [65]. In neuroblastoma tumors, lack of Thy-1 expression correlates with reduced patient survival [49].

Patients with GO display an increase in volume of the extraocular muscles and/or the intraorbital adipose tissues. As in IPF, fibroblasts are implicated in the pathology of this disease [66] and Thy-1 expression delineates differentiation potential. Unlike in IPF, only Thy-1 (+) orbital fibroblasts appear capable of differentiating into myofibroblasts, whereas Thy-1 (−) are incapable of doing so but are unique in their ability to differentiate into mature adipocytes [10].

Intraorbital adipose tissue from patients with GO has a greater proportion of Thy-1 (+) fibroblasts relative to that taken from healthy patients [52]. This seemingly conflicting modulation of differentiation potential by Thy-1 and its involvement in GO is poorly understood and requires further study. Thy-1 fibroblast heterogeneity has also
been noted in the human myometrium, in which the Thy-1 (+) subset also differentiates in to myofibroblasts, and in which there are differences in expression of cyclooxygenase isoforms and MCP-1 [9], [10], [67].

Psoriasis is a common chronic inflammatory skin disease in which the infiltration of neutrophils is an important feature of pathogenesis. Thy-1 is involved in the adhesion of neutrophils and monocytes to activated microvascular endothelial cells via interaction with the β2-leukocyte integrin Mac-1 (CD11b/CD18). The enhanced adhesion of psoriatic neutrophils to Thy-1-expressing endothelial cells, via Mac-1/Thy-1 interaction, suggests that this may be an important mechanism of attachment and migration into psoriatic lesions [68].

Given the possible role of Thy-1 in multiple pathogenic alterations, as well as the contrasting phenotypes and activities exhibited between Thy-1 (+) and (–) cells in different tissues, it is useful to consider carefully the mechanisms by which Thy-1 is regulated in evolution and development, and dysregulated in certain disease states. 3.

3. Thy-1 structural evolution and species differences

Thy-1 belongs to the immunoglobulin superfamily [17] and is evolutionarily conserved; significant homology exists among multiple species including squid, chicken, frogs, mice, rats, and humans. Additionally, it has been proposed that the immune system is evolutionarily related to the nervous system, and Thy-1, which is an important molecule in both, possibly represents a primordial domain of the immunoglobulin superfamily ancestry [68]. As the mouse is the predominant in vivo mammalian
biological model, there is considerably more information about genetic regulation and structure of murine Thy-1. The \textit{thy1} locus is mapped to mouse chromosome 9, at which there are two alleles that encode the proteins designated Thy-1.1 and Thy-1.2. The two are distinguished by a single AA at position 89, arginine and glutamine, respectively. In humans, THY1 is mapped to chromosome 11q22.3 and initially expressed in a 161 AA pro form but undergoes several post translational modifications [19]. The first 19 AA of Thy-1 act as a signal peptide that targets it to the cell membrane and is later removed (Fig. 2). Thy-1 is also N-glycosylated at two [20] to three sites [18], [21]; carbohydrate content accounts for nearly 30% of its mass, which ranges from 25 to 37 kDa [21]. Between different tissues, the carbohydrate moiety composition may vary dramatically [22], [23]. Thy-1 is initially kept at the cell surface by AA 132–161, which embed into the membrane. However this C-terminal transmembrane domain is cleaved away and a GPI moiety is added at residue 131. The GPI moiety is composed of two fatty-acyl groups that tether Thy-1 to the cell surface and participate in targeting to lipid rafts [20]. Thy-1 is differentially expressed and distributed among many species and among tissues of the same species. In mice, it is expressed on the surface of various cells including thymocytes, T-lymphocytes, bone marrow stem cells and in high levels in neurons and some fibroblasts. In the thymus, Thy-1 is the most abundant glycoprotein expressed on the surface, covering 10–20% of thymocyte surface area [69]. In humans, Thy-1 is absent from mature T cells, but expressed on a subset of CD34+ bone marrow cells, and umbilical cord blood- and fetal liver-derived hematopoietic cells. The highest expression of Thy-1 in humans is found on (primarily fetal) thymic stromal cells and in most fibroblasts. In bone marrow and in circulating leukocytes, Thy-1 is present in a
small proportion of cells, primarily in a subset of CD34+ and CD3+ CD4+ lymphocytes. Thy-1 is also expressed in endothelial cells, smooth muscle cells, some leukemic and lymphoblastoid cells, such as THP-1 [70].

Thy-1 is one of the most highly glycosylated membrane proteins with a carbohydrate content up to 30% of its molecular mass [18]. The composition of Thy-1 carbohydrate moieties varies between different tissues or cells of the same lineage and among cells in different stages of differentiation. For example, in rats sialic acid in thymic Thy-1 far exceeds that found in brain Thy-1 and galactosamine is found only in brain Thy-1 [18]. In contrast to the Thy-1 antigen of most other species, guinea pig Thy-1 has a much higher molecular weight, which is due to a more extensive N-linked glycosylation, bringing the molecular radius up to 36kDa [71].

4. Soluble Thy-1

Thy-1 does not exist solely in a membrane bound form. A soluble form of Thy-1 has been detected in serum, CSF, wound fluid from venous leg ulcers, and synovial fluid from joints in rheumatoid arthritis [21], [28]. Speculated methods by which cells produce soluble Thy-1 include an alternative mRNA splice variant omitting addition of the GPI anchor, or an enzyme that cleaves Thy-1 away from the cell surface. Interestingly, the glycosylation pattern of soluble Thy-1 can differ from that of its presumptive source. For example, soluble Thy-1 detected in CSF has slightly higher MW than Thy-1 in cerebral cortex membranes. This differential size is attributed to unique glycosylation patterns; specifically, soluble Thy-1 is resistant to Endo H, which indicates low mannose content,
whereas Thy-1 in cerebral cortex membranes is Endo H susceptible. However, both the soluble and membranous forms are identical in size when all the N-glycosylated groups are removed [21]. Soluble Thy-1 in CSF could possibly originate from a region of the brain other than the cerebral cortex. In rats, the carbohydrate moiety of brain Thy-1 has considerably less sialic acid content than that of thymocytes [22], though variation between regions of the brain is not well characterized.

These findings suggest that the GPI anchor is severed in close proximity to the protein moiety, for example, within the GPI moiety itself or at a protease target just up stream. As for targeting within the GPI moiety, both GPLD1 and PI-PLC can do so. The former is produced in mammals whereas the latter is of bacterial origin. These enzymes have distinct enzymatic requirements to cleave Thy-1 from the cell surface. Detergents or saponins are needed by exogenous GPI-PLD to cleave Thy-1, whereas PI-PLC does not have such a requirement [35]. Although detergents are not required by PI-PLC, the susceptibility of Thy-1 to cleavage varies form one cell type to another [35]. This is relevant given that serum contains GPI-PLD and as a consequence Thy-1 positive endothelial cells as well as circulating T-cells are susceptible, yet the concentration of serum Thy-1 is only 251 ± 105 ng/mL [28]. Localization of Thy-1 to cholesterol rich lipid rafts is thought to protect it from GPI-PLD present in serum [26]. Release of Thy-1 could also result from proteolysis, which may be an important mechanism in fibroblasts given the large number of proteolytic enzymes produced by fibroblasts [72], [73] and the aforementioned protection from GPI-PLD activity afforded by its location within its native membrane microdomain. Shedding of cell surface receptors is a common means of modulating their activity. For example, the a disintegrin and metalloproteases are known
to release a plethora of surface proteins including those tethered by GPI anchors [74]. The exact mechanism(s) of Thy-1 shedding and possible roles of shedding in normal biology and in disease have yet to be determined. Regardless of the means, shedding of Thy-1 could very likely play an important role in facilitating its complete removal from the cell surface, given its unusually slow turnover rate [36].

Cultured lung fibroblasts shed Thy-1 into the media when treated with various profibrotic cytokines, such as IL-1β and TNF-α [15], yet the soluble and membrane bound Thy-1 have indistinguishable migration speeds through an acrylamide gel (unpublished observation). In contrast to fibroblasts, endothelial cells (ECs) increase their expression of Thy-1 when stimulated with profibrotic cytokines [75], [76]. The leukocyte integrin Mac-1 interacts specifically with Thy-1 expressed on activated ECs, which suggests a role for Thy-1 in recruiting leukocytes into areas of inflammation [77]. Additionally, αvβ3 integrin expressed on melanoma cells interacts specifically with Thy-1 expressed on activated ECs. This is of particular relevance given that αvβ3 integrin expression by melanoma cells is closely associated with tumor progression and metastases formation in melanoma [78]. Thy-1−/− mice have an impaired cutaneous immune response. Because wild type (WT) thymocytes express high levels of Thy-1 and Thy-1−/− thymocytes do not properly mature, it was suggested that the impaired cutaneous immune response was a consequence of defective fine-tuning of T cell effector functions [61]. Alternatively, or in conjunction with this postulated mechanism, absence of Thy-1 on activated endothelial cells may account for the impaired cutaneous immune response. Regardless, it is evident that regulating Thy-1 expression at the sites of inflammation is of vital importance to
resolving tissue injury. As of yet, it is unknown whether Thy-1 in itself has a role in its own regulation.

The biological functions of soluble Thy-1 remain unclear. It is important to note that Thy-1 lacking the GPI anchor very often becomes unrecognizable by antibodies against its membranous form [37]. Shedding of Thy-1 could potentially elicit a number of effects. Fewer Thy-1 molecules at the cell surface could directly affect the cell by limiting ligand binding and signal transduction. Soluble Thy-1 could also retain a comparable affinity with its ligand to that of its membranous form, making ligand unavailable to Thy-1 at the surface of the cell from which it was shed as well as on neighboring cells. In the human uterine cervix, Thy-1- expressing vascular pericytes appear to secrete Thy-1- intercellular vesicles which communicate with basal epithelial cells as part of the “tissue control unit” of mesenchymal-epithelial interaction [79]. The biological significance of this intriguing phenomenon is uncertain.

5. Transcriptional regulation of Thy-1 mRNA

The unique expression profile of Thy-1 is reflective of unusual regulatory elements that govern it (Fig. 3). The Thy-1 promoter has two elements that are traditionally attributed to “housekeeping” genes; specifically it is located within an area of high G/C content and has no classical TATA box [80], [81]. In addition, Thy-1 transcription initiates at multiple sites, and distribution of these sites differs in murine brain and thymus [82]. Transgenic mice with a hybrid or deletion construct of the Thy-1 transcriptional unit were found to have tissue-specificity control elements exclusively
downstream of the cap site. Thy-1 expression in the mouse thymus and brain requires sequences located in intron 3 and at the 3′ end of intron 1, respectively. These downstream elements function independently of each other, as deletion of intron 3 eliminates expression in the thymus whereas levels in the brain are unaffected. Within the third intron of murine \textit{thy1} is a 36 bp region that is capable of specifically binding an Ets-1-like nuclear factor expressed by both mouse thymocytes and splenocytes. Accordingly, Thy-1 is expressed in both mouse cell types. In the rat, however, the corresponding region differs by only three nucleotides and is incapable of binding a similar Ets-1-like nuclear factor in rat thymocytes. However, the region does recognize another nuclear factor expressed by rat thymocytes but not splenocytes. Unlike in mice, rat splenocytes do not express Thy-1. These data suggest that Thy-1 expression in rat thymocytes and not splenocytes is due to the conserved 36 bp recognizing a nuclear factor found in the former and not in the latter [44]. This differential tissue expression between species as closely related as rat and mouse exemplifies the unique context-dependency of Thy-1 regulation and the difficulty in making broad inferences regarding its biology as it relates to disease states.

Deletion of intron 1 eliminates expression in the brain whereas levels in the thymus are unaffected. Tissue-specific transcription of Thy-1 requires those cis-acting sequences within the introns to cooperate with at a minimum 300 bp (−270 to +36) of the promoter. However, replacement of the Thy-1 promoter with another heterologous promoter does not abolish the tissue-specific or developmental expression profile of Thy-1 [83]. A murine \textit{thy1.2} genomic expression cassette has been designed to drive expression in the nervous system. The cassette is void of all Thy-1.2 coding sequences
and the thymus enhancer in intron 3, but retains the neural enhancer element in the first intron [84].

The endogenous Thy-1 promoter in itself is not sufficient to elicit transcription or tissue specificity without the downstream elements [83]. This makes the promoter unique for the “monogamous” relationship with its endogenous gene. Two transcription factors, Sp1 and CP1, are known to bind the Thy-1 promoter and are indispensible to its transcription in vivo. Three additional proteins, distinct from Sp1 and CP1, bind the promoter and were given the designation R1, R2, and R3 [81]. As of yet, the identity of these proteins remains unknown.

6. Post-transcriptional regulation of Thy-1 mRNA

Though a far less examined mechanism, early evidence suggests that post-transcriptional regulation of Thy-1 mRNA determines the temporal expression of the Thy-1 protein in specific areas of the developing mouse nervous system. Expression of Thy-1 mRNA in these areas can precede detection of Thy-1 protein by several days. As mentioned previously, transcription of Thy-1 mRNA can occur at different initiation sites. However, the onset of protein expression does not coincide with any change to the size or transcription initiation site of Thy-1 mRNA, rather it appears to be the result of a yet to be elucidated post-transcriptional mechanism [85].

Heterokaryons generated from the fusion of mature Thy-1.1 expressing neurons with immature Thy-1.2 negative neurons became Thy-1 negative within 16 h of fusion. However, these heterokaryons behave in a similar manner as cultured immature Thy-1.2
negative neurons in that Thy-1.2 expression becomes evident within 3–4 days in culture and also coincides with the re-expression of the Thy-1.1. As the nuclei of the Thy-1.1 and Thy-1.2 neurons are distinct within the heterokaryon, the inhibition of Thy-1.1 expression was concluded to be the consequence of a developmentally regulated diffusible suppressor molecule [86]. This lends support to developmental regulation of Thy-1 in the nervous system being, at least in part, a post-transcriptional event. Therefore unlike fibroblast and endothelial cells for which there is evidence for cytokine involvement in Thy-1 regulation, Thy-1 regulation in developing neurons seems to be the consequence of intrinsic factors.

7. Genetic and epigenetic regulation of Thy-1

The field of epigenetics encompasses regulation of gene expression that is often heritable but does not involve changes to DNA sequences. Two mechanisms by which this occurs include post-transcriptional modifications to histones and methylation of DNA within cytosine-guanine (CpG) islands [87]. As stated previously, Thy-1 acts as a tumor suppressor in nasopharyngeal cancer and can be down regulated by methylation of its promoter [51].

In both rat and human primary lung fibroblasts, CpG islands in the Thy-1 gene promoter are hypermethylated in the Thy-1 negative fibroblast subpopulation but not in the positive. The absence of Thy-1 results in part from promoter hypermethylation. In keeping with this finding, 5-aza-2’-deoxycytidine, a DNA methyltransferase inhibitor, induces Thy-1 expression in Thy-1 (–) cells. Moreover, methylation-specific PCR-in situ
hybridization in lungs of patients with IPF demonstrated Thy-1 promoter hypermethylation within FF, which are populated with predominantly Thy-1 (−) myofibroblasts [48]. These findings suggest that epigenetic silencing of Thy-1 may be a pathogenic mechanism in IPF, as has been suggested for nasopharyngeal carcinoma.

**Concluding remarks**

Thy-1 as a biofactor is remarkable both in the number of cell types expressing it and the diversity of phenotypes associated with its expression. It is expressed in hematopoietic and stromal stem cells in a relatively undifferentiated state, whereas in neurons, Thy-1 is associated with maturation and cessation of neurite outgrowth. In fibroblasts, Thy-1 expression accompanies specific phenotypes which depend upon the tissue from which they originate. Absence of Thy-1 in lung and synovial fibroblasts indicates a more fibrotic myofibroblast phenotype, whereas the opposite may be the case for orbital and myometrial fibroblasts. As of yet, much is still unknown regarding the exact mechanisms by which Thy-1 modulates these phenotypes and by which Thy-1 itself is regulated. What is clear is that both the roles and regulation of Thy-1 are context-dependent. The regulation of Thy-1 is a promising area of research; increased understanding of the mechanisms of Thy-1 regulation may lead to the possibility of therapeutic manipulation of cellular phenotypes in such diverse fields as nerve injury, cancer and fibrosis.
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Fig. 1. Thy-1 and fibroblast phenotype in pulmonary fibrosis. Fibroblasts in fibroblastic foci, the hallmark lesion of IPF, lack Thy-1 expression. Evidence exists for shedding of soluble Thy-1 (sThy-1) and epigenetic silencing of Thy-1 expression. Thy-1 (2) lung fibroblasts demonstrate increased migration, myofibroblastic differentiation, activation of latent TGF-β, and resistance to apoptosis. The absence of Thy-1 expression in pulmonary fibroblasts thus promotes a fibrogenic phenotype.
Fig. 2. Thy-1 molecule and proposed soluble forms. Thy-1 is initially generated as a 161 AA pro form. The initial 19 AA signal peptide is removed, and the terminal 29 AA are replaced with a GPI anchor, generating the mature form, which is anchored to the outer leaflet of the cell membrane by the diacyl group of the GPI anchor. Post-translational modifications are not shown. Shed Thy-1 could be generated either by cleavage of the GPI anchor by GPLD1, or by undefined proteases acting at as-yet undetermined cleavage sites.
Fig. 3. Murine Thy-1 gene structure and control elements. There are four exons and three introns. There are Sp1 and CP1 binding sites in the promoter region. Methylation of a CpG island in the first intron (*) can result in transcriptional silencing in rat and human fibroblasts. There are sequences conferring tissue specificity for brain and thymus in the first and third introns, respectively.
References


EFFECT OF THE GPI ANCHOR OF HUMAN THY1 ON ANTIBODY RECOGNITION AND FUNCTION

by

JOHN E. BRADLEY, JAMES S. HAGOOD, JOY M. CHAN

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ABSTRACT

Thy-1 is a GPI-linked cell surface glycoprotein expressed on the surface of numerous cell types, which regulates signals affecting cell adhesion, migration, differentiation, and survival. In addition, THY1 has been detected in serum, cerebral spinal fluid, wound fluid from venous ulcers, synovial fluid from joints in rheumatoid arthritis and more recently urine. Soluble and membranous forms of THY1 from in-vivo sources have been shown to be identical in size when all the N-glycosyl groups are removed. These findings suggest that soluble THY1 is separated from the diacyl portion of its GPI-anchor by hydrolysis of a bond within the GPI moiety. For Thy-1 and other GPI-anchored proteins, the general consensus is that delipidation induces a stable change in conformation that manifests itself in a change in antibody affinity. Moreover, this phenomenon has been observed for GPI-anchored proteins expressed as recombinant soluble forms. Using epitope tagged recombinant soluble THY1, we report that widely available monoclonal antibodies to human THY1 from clones K117, 5E10, and AS02 are unable to detect soluble forms of THY1 by immunoblotting. We reevaluated the solubility of THY1 that we previously reported detecting in the CM of NHLFs and found it to be predominantly insoluble. Taken together, these findings suggest that monoclonal antibodies used to detect THY1 in body fluids are unlikely to detect delipidated, truly soluble forms. This phenomenon should be considered in the generation of antibodies and controls for THY1 bioassays. Furthermore, the change in THY1 conformation with delipidation, beyond affects antibody affinity, may affect its function.
INTRODUCTION

Thy-1, a GPI-linked cell surface glycoprotein on stem cells and multiple mature cell types, was originally discovered in an attempt to raise antiserum against leukemia-specific antigens from the C3H mouse strain in the AKR mouse strain and vice versa. The antibodies were found to strongly label thymocytes as well as peripheral T cells [16]. For this reason, the original designation for the antigen changed from Theta to Thy-1 [42]. The cell types expressing Thy-1 in normal and pathological conditions, and the immunologic and non-immunologic roles of Thy-1 have been reviewed elsewhere [13], [41], [88].

Our lab previously reported that NHLF treated with several different pro-inflammatory cytokines undergo a decrease in the level of cell surface THY1 expression. This decrease coincides with an increase of detectable THY1 in the CM, suggesting release of THY1 from the cell surface. From these observations, we concluded that THY1 is likely separated (shed) from the diacyl portion of its GPI-anchor by a phospholipase hydrolyzing a bond within the GPI moiety [15]. Others have demonstrated Thy-1 detectable in serum, wound fluid, urine and CSF in a variety of normal and pathological conditions [21], [28], [39]. Taken together, these findings indicate that released THY1 may serve as a useful biomarker for certain pathological conditions. In addition, recent studies have shown that a soluble recombinant THY1 in which the GPI-attachment signal is replaced by the Fc fragment of human IgG1 (THY1-Fc) alters activation of latent TGF-β and cell phenotype in lung fibroblasts [89], indicating that THY1 may have a role as a soluble mediator in addition to its function on the cell
surface. For these reasons, antibody detection of THY1 in biological fluids may be important in clinical or research applications.

Antibody mediated detection or purification of THY1 can be problematic for several reasons. Many of the monoclonal antibodies employed to detect Thy-1 in western blots require it be prepared under non-reducing (NR) conditions, including OX7, G7, HO-13-4, 5E10, and ASO2 [37–39], [90]. For many antibodies that recognize GPI-anchored proteins at the cell surface, their affinity is lost or greatly diminished if that same protein is delipidated. This phenomenon has been reviewed elsewhere [91]. For Thy-1 in particular, several monoclonal and polyclonal antibodies to mouse Thy1 have been shown to have this caveat [37], [90]. Though mostly demonstrated for antibodies to mouse Thy1, a group reported the reactivity of an antiserum raised against membrane-bound human THY1 was completely negative toward hydrophilic human THY1 purified from CSF. For Thy-1 and GPI-anchored proteins, the general consensus is that delipidation induces a stable change in conformation that manifests itself in a change in antibody affinity [91]. Antibodies that recognize human THY1 with a GPI anchor are predicted to have lower affinity for THY1 if it is delipidated.

The monoclonal antibodies K117 (American Type Culture Collection [ATCC] Number: HB-8553), 5E10 (STEMCELL Technologies 01437), and AS02 (Millipore CP28) were generated from mice immunized with a human astrocytoma cell line, a human erythroleukemia cell line, and human dermal fibroblasts, respectively. The antigen recognized at the cell surface by K117, 5E10, and AS02 is THY1. We performed the following studies to characterize recognition of soluble and GPI-anchored forms of THY1 by antibodies from these clones.
MATERIALS AND METHODS

Recombinant Constructs of THY1

For expression of WT human THY1, the complete cDNA of human THY1 (AAH65559.1) was ligated into the mammalian expression vector pcDNA3.1/Zeo (+) (Invitrogen V860-20) with the Kozak sequence GCCGCC [92] just upstream of the start codon. The restriction sites EcoRI and NotI were used at the 5′ and 3′ end, respectively [Figure 1. A and B]. To express mature THY1 with an N-terminal FLAG tag, a coding sequence for the FLAG epitope [93], [94] was cloned immediately downstream of that of the ER localization signal [95] and just upstream of the first codon for mature THY1 [Figure 1. A and B]. To express THY1 with its GPI-attachment signal replaced with that of another glycoprotein, coding sequences for the foreign GPI-attachment signal were cloned downstream of a “hinge” region. This “hinge” region comprises the 6 AA downstream of mature THY1, EGISLL, changed to GGIGLS as was previously shown to successfully add the transmembrane domains of CD8 and NCAM to mouse Thy1 [58]. Using site-directed mutagenesis, an intermediate construct was made first by changing the sequence just downstream of the codons for mature THY1 to code for GGIG followed by the BstBI restriction site. Expression vectors containing the cDNA of the GPI-attachment signals for DAF and TRAIL-R3 were kindly provided by Daniel F. Legler (University of Konstanz, Konstanz, Germany). The cDNA of TRAIL-R3 contains multiple threonine, alanine, proline, and glutamine-rich repeats that present multiple annealing sites for the 5′ cloning primer. To avoid this possibility, the TRAIL-R3 containing expression vector was cut with PvuII and NotI producing a 115 bp fragment. This fragment was used as the template for PCR. PCR products of TRAIL-R3 and DAF
had the restriction sites of BstBI and NotI introduced 24 base pairs upstream of the final codon in the mature protein and after the stop codon, respectively. These PCR products were cloned into the intermediate construct with BstBI and NotI. Finally, the BstBI sites in both were converted to codons for LS by site directed mutagenesis [Figure 2. A and B]. To express sTHY1 with a C-terminal histidine tag, an intermediate construct was made introducing half the changes required for six histidines then a stop codon to follow the “hinge” region by site-directed mutagenesis. The intermediate was then used as the template in another round of site directed mutagenesis to complete the required changes [Figure 2. A and B]. To express soluble THY1 with an N-terminal FLAG tag, the “hinge” region was introduced followed by a stop codon into FLAG – THY1 by site directed mutagenesis [Figure 1. A and B]. See Figures 1. and 2. for a schematic of these constructs.

**Cell Culture of RFL-6 and CCL-210**

Rat fetal lung fibroblasts (RFL-6) (ATCC) were maintained in F-12K supplemented with 10% FBS, 1% penicillin, and 1% streptomycin with medium exchanged every 2 days. CCL-210 NHLF (ATCC) were maintained in MEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin with medium exchanged every 2 days. Both were passaged using 0.25% trypsin. Cell extracts (CE) and CM were collected in the following manner. Adherent cells were washed twice with PBS then harvested by scraping into ice cold IP lysis buffer (Pierce 87788). Cell lysates were incubated on ice for 5 min with periodic vortexing then centrifuged at 13,000 × g and 4°C
for 10 min. The supernatant containing the CE was stored at -80°C prior to use. CM from adherent cells was cleared of cellular debris by centrifugation at 1,200 × g and 4°C for 5 min. The supernatant was either immediately concentrated using Amicon’s Ultra-2 Centrifugal Filter Unit with a 3kDa retention membrane, precipitated with methanol, submitted to differential centrifugation, or stored at -80°C. For cytokine treatment, cells at 90% confluency were washed with serum free media (SFM) then serum starved in SFM for 24 hrs, followed by culture in either MEM alone or 20ng/mL each TNFα (GIBCO PHC3015L) and IL-1β (GIBCO PHC815) in MEM for 24 hrs to 48 hrs.

Stable Expression of Recombinant THY1 in RFL-6

RFL-6 were transfected using Lipofectomine 2000 (Invitrogen 11668) according to the manufacturer’s protocol. After 24 hrs, cells were subcultured in growth media so as to be near 10% confluent the following day, then washed with SFM and cultured in growth media supplemented with 500µg/mL Zeocin (Invitrogen). Stably transfected cells were selected after several passages showed no visual indication of cell death.

Deglycosylation of THY1 with PNGase F

CE or CM were diluted in water and brought to 1% SDS with a 10% stock solution then incubated at 100°C for 10 min., following the manufacturer’s standard protocol for PNGase F (New England BioLabs P0704) except for omission of dithiothreitol from the denaturing buffer. An aliquot was taken as the control for THY1
prior to deglycosylation. G7 reaction buffer and NP40 were added to a final concentration of 1X and 1%, respectively. After adding PNGase F, this reaction mixture was incubated at 37°C for 1 hour then diluted with PBS to the same total protein concentration.

*Release of THY1 from Cell Surfaces with PI-PLC*

Cells expressing THY1 were cultured in growth media to near 90% confluency, washed with SFM, then incubated with 0.1U/mL PI-PLC (Invitrogen P-6466) in SFM for 24 hrs.

*Analysis of Cell Surface THY1 Expression by Flow Cytometry*

Flow cytometry was performed as previously described [15] using the following antibodies: anti-human THY1 and mouse IgG1 κ isotype control conjugated to FITC (BD Pharmingen 555595 and 555748, respectively).

*Western Blot Analysis*

Unless otherwise specified, all samples were prepared in NR SDS loading buffer (50mM Tris-HCl pH 6.8, 2% SDS, and 10% glycerol). Sample preparations were electrophoresed in 10% polyacrylamide gels then transferred onto PVDF membranes. Depending on the primary antibody, membranes were either blocked in 5% Milk in TBST or TBST alone. The primary antibodies for detecting the mature THY1
polypeptide, K117, 5E10, and AS02 were diluted to 0.1µg/mL in TBST. The primary antibodies for detecting the FLAG (Sigma-Aldrich M2 F1804) and 6 histidine epitopes (Rockland 600-401-382) were diluted to 1.0µg/mL in 5% Milk in TBST. All primary antibodies were incubated with membranes overnight at 4°C. Antibodies bound to antigen were visualized with secondary antibodies conjugated to horseradish peroxidase (HRP) in conjunction with a chemiluminescent substrate. The secondary antibodies used were goat anti-mouse (H+L) – HRP (Bio-Rad 172-1011) and goat anti-rabbit (H+L) – HRP (Thermo Scientific 32460) diluted 1/200,000 and 1/40,000 in blocking buffer, respectively. When required, membranes were stripped with Pierce’s Restore western blot stripping buffer following the manufacturer’s protocol.

**Partitioning of CM using Triton X-114**

Triton X-114 (Sigma-Aldrich 93422) was added to concentrated CM to a final concentration of 2%. An aliquot was taken as the control for total THY1 prior to partitioning. With periodic vortexing throughout, the remainder was kept on ice for 10 min, then another 10 min at 37°C. Following this, the sample was immediately centrifuged at 21,000 × g for 10 min at room temperature. Centrifuging produced a clear partition between the insoluble and soluble phase. Prior to western blot analysis, volumes were adjusted to represent equal fractions of starting volume of CM.
Differential centrifugation and Methanol Precipitation of CM

Two 150µL aliquots were taken from the supernatant of CM centrifuged at 1,200 × g and 4°C for 5 min. One aliquot was centrifuged again at 21,000 × g for 5 min at 4°C. After this, the supernatant was removed. All samples were precipitated by adding 1,350µL of methanol, then leaving them at -20°C overnight. The following day all were centrifuged at 21,000 × g for 30 min at 4°C. The supernatant was poured off and the precipitant was allowed to air dry before being resuspended in 35µL of 1X SDS loading buffer then submitted to western blot analysis.
RESULTS

Anti-human THY1 monoclonal antibodies which react with THY1 at the cell surface do not recognize delipidated THY1

Human THY1 is readily detected by western blot in NR conditions using either K117, 5E10, or AS02 monoclonal antibody [Figure 3. A and B and Supplementary Figure 1.]. The NHLF cell line CCL-210 was treated with PI-PLC as described in Methods. Analysis by flow cytometry showed THY1 was completely removed by PI-PLC (data not shown). However, THY1 was not detected in the CM of PI-PLC treated CCL-210 using the K117 monoclonal antibody, despite being easily detected in CE diluted to the same volume as the CM (data not shown).

In order to confirm the presence of THY1 released by PI-PLC into CM, a recombinant form was engineered to be expressed at the cell surface with an N-terminal FLAG tag. This was accomplished by introducing the FLAG epitope [93], [94] downstream of the N-terminal ER localization signal [95] and just upstream of the first residue of mature THY1, designated FLAG – THY1 [Figure 1 A and B]. The size of FLAG – THY1 stably expressed by RLF-6 relative to WT THY1 is in keeping with the predicted polypeptide mass difference of ~1.3kDa and being correctly glycosylated [Figure 3A]. CE from RFL-6 stably expressing FLAG – THY1 and the CM of the same cell line treated with PI-PLC treated were compared in western blots with anti-FLAG as the primary antibody (data not shown). Using FLAG band intensity to control for loading of THY1, CM from PI-PLC treated fibroblasts were used in western blots with clone K117 5E10, and AS02 as the primary antibodies. Each western blot showed a band in the lane corresponding to CE but not the CM [Figure 3. B and Supplementary Figure 1.]

After stripping the membranes of primary and secondary antibodies for detecting THY1,
each was re-probed with FLAG antibody. Unlike with clones K117, 5E10, and AS02, the immunoblot for FLAG revealed a band in each lane equal in size. However, the band appearing in the lane in which PI-PLC released FLAG – THY1 was run was equal or greater in intensity as predicted [Figure 3.B]. Thus, sufficient FLAG – THY1 was present for clones K117, 5E10, and AS02 to detect THY1 were it not delipidated.

**Monoclonal antibodies from clones K117 and 5E10 recognize epitopes on THY1 independent of its glycosylation, but are abolished under reducing conditions**

Glycosylation of THY1, which is entirely N-linked, can account for more than 50% of its total mass. Between different cell and tissue types, the carbohydrate moiety composition may vary dramatically. As a consequence, the molecular mass of THY1 ranges from 25 to 37kDa [21], [23], [38], [90]. Of the anti-mouse monoclonal antibodies that have affinity for non-delipidated Thy1, OX7, H140-150, H154-177 [90], and AS02 [38] have been demonstrated to recognize their respective epitopes independent of glycosylation. Therefore, the epitopes lost with delipidation are likely confined to a region of the Thy-1 polypeptide. The mature THY1 polypeptide has four cysteine residues. As a member of the immunoglobulin superfamily, each cysteine is predicted to form a disulfide bond with one of the other under oxidizing conditions [96]. Western blots to detect Thy1 that utilize antibodies from clones HO-13-4, G7 [37], OX7 [90], 5E10 [39], or AS02 [38] as the primary are conducted under NR conditions. NR conditions are used with clone K117 as well [Figure 3].

To determine if recognition by clones K117 and 5E10 are contingent on glycosylation or disulfide bonds, both were used as the primary antibodies in western
blots of native, reduced and completely deglycosylated THY1 in CCL-210 extract. PNGase F cleaves between the innermost GlcNAc and asparagine of oligosaccharides from N-linked glycoproteins and was shown to completely remove the glycans from human THY1 [21]. With clone K117 [Figure 4] and 5E10 (data not shown), a single band of a size corresponding to fully glycosylated THY1 was detected in the lane containing non-PNGase F treated CE prepared under NR conditions. To circumvent possible diffusion of the reducing agent into adjacent lanes, samples were run in western blots with an empty lane between those with reducing agents and those without [Figure 4]. No bands were detected in the lanes containing non-PNGase F treated cell lysate prepared under reducing conditions. A single band of a size corresponding to completely deglycosylated THY1, ~12.5 kDa, was detected in the lane containing PNGase F treated CE prepared under NR conditions. Moreover, the bands were equal in intensity and relative size [Figure 4]. As is the case with clones OX7, H140-150, H154-177, and AS02, the epitopes recognized by K117 and 5E10 on human THY1, which are lost with delipidation, remain with deglycosylation. Additionally, breaking disulfide bonds with reducing agents completely abolishes epitope recognition by these antibodies.

*Substitution of the native GPI anchor of THY1 with the GPI-attachment signals of DAF or TRAIL-R3 does not alter antibody reactivity despite the presence of an intervening 15 AA “hinge” region*

Recombinant THY1 hybrids were engineered to have GPI anchors attach using the DAF and TRAIL-R3 GPI attachment signals, designated THY1 – GPI(DAF) and THY1 – GPI(TR3). To accomplish this, a “hinge” region was placed between mature
THY1 and the foreign GPI-attachment sequences. The hinge region is based on prior publication by a group that successfully replaced the GPI-attachment signal of mouse Thy1 with the transmembrane domain of CD8 and NCAM [58]. Both GPI attachment signals consist of the C-terminus 8 residues upstream of the GPI anchorage sites of DAF and TRAIL-R3 [Figure 2. A and B].

THY1 is detected in RFL-6 cells transfected with expression vectors for THY1 – GPI(DAF) or THY1 – GPI(TR3) as assessed by western blot using clone K117 [Figure 3. A]. The bands for each are the same size and in keeping with the predicted polypeptide mass difference of ~1.3kDa relative to the band for WT THY1 and are correctly glycosylated. Identical results were obtained using clone 5E10 (not shown). After 24 hr incubation with PI-PLC, cells expressing THY1 – hinge – GPI(DAF) and – GPI(TR3) are completely negative for cell surface THY1 as assessed by flow cytometry. This confirms that both are GPI anchored and neither GPI-anchor possesses an additional palmitoyl group on the inositol. PI-PLC hydrolyzes the bond in the GPI anchor that liberates the diacylglycerol but not the palmitoyl group from the inositol [97]. For both, the GPI anchor is predicted to attach 15 AA downstream of the WT attachment site of THY1 [Figure 2. B]. This is confirmed by the size of each relative to native THY1 and recombinant WT THY1 [Figure 3. A]. Thus, GPI anchors preserve the conformation required for recognition of THY1 by K117 and 5E10 even when they are up to 15 AA removed from the WT attachment site. Moreover, a GPI anchor attached to THY1 by a non-endogenous GPI anchor attachment signal can confer a conformation recognizable by K117 and 5E10.
Recombinant soluble THY1 is not recognized by the anti-human THY1 monoclonal antibodies from clones K117, 5E10, and AS02

Recombinant soluble THY1 without a GPI anchor can be expressed in mammalian cells by introducing a stop codon downstream or in place of the codon for CYS 130 and upstream of the GPI anchor attachment signal [28], [95]. Without a tether to the inner leaflet, recombinant THY1 in the ER traffics to the Golgi, then into the CM. This approach was taken in the design of recombinant sTHY1 [Figure 1. A-B and 2. A-B].

Two bands, approximately 25 and 20kDa in size, are detected in the CM of RFL-6 transfected with expression vectors for FLAG – sTHY1 and sTHY1 – 6X HIS. The slower migrating band is always a greater intensity than the smaller band and in keeping with the predicted polypeptide mass difference, lacking a GPI anchor, and being correctly glycosylated [Figure 5. A and B]. To determine if the two bands represent differentially glycosylated recombinant sTHY1, western blots were performed using sTHY1 in CM treated with PNGase F. With PNGase F treatment, the CM no longer contained the ~25 and ~20kDa bands but rather a single band between 15 and 10kDa [Figure 5. B]. The predicted molecular mass of the polypeptide alone is ~13.9kDa. N-terminally tagged sTHY1 was used to confirm that sufficient recombinant sTHY1 is present in CM of transfected cells for K117, 5E10, and AS02 to be able to detect it, if it maintained the correct conformation without a complete GPI anchor. CE from RFL-6 cells expressing FLAG – THY1- and FLAG – sTHY1-containing CM were run in western blots with anti-FLAG as the primary antibody. The relative concentration of THY1 in these preparations was determined in the same manner as before by assuming FLAG band intensity is directly proportional to the concentration of THY1. Sample preparations of CE and CM
were loaded so a greater amount of FLAG – sTHY1 was run in western blots with clone K117, 5E10, and AS02 as the primary antibody. As with FLAG – THY1 delipidated by PI-PLC, both western blots showed a band in the lane corresponding to the cell lysates but not the CM [Figure 3. B]. After stripping the membranes of primary and secondary antibodies for detecting THY1, each was re-probed with FLAG antibody. As anticipated, there was more FLAG – sTHY1 in the lanes containing the CM [Figure 3. B].

Unlike the case with anti-human THY1 monoclonal antibodies, recognition of the histidine epitope at the C-terminus of sTHY1 – 6X HIS requires or at least is greatly facilitated with reducing conditions [Figure 5. A]. Reduced and non-reduced sTHY1 – 6X HIS run in adjacent lanes reveals a ~25 kDa band that spans the entire lane of the former and at the edge of the latter [Figure 5. A]. Even after concentrating several fold, non-reduced sTHY1 – 6X HIS was never detected by western blot with K117 (data not shown).

**The increased THY1 in the CM of normal human fibroblasts treated with pro-inflammatory cytokines, that coincides with a decrease at the cell surface, is insoluble**

The monoclonal antibody from clone K117 detects basal and cytokine induced increases in levels of THY1 in the CM of CCL-210. However, as demonstrated in Figures 3. B, K117 does not recognize sTHY1 expressed without a GPI-anchor or THY1 delipidated with PI-PLC. This suggests that THY1 detected in the CM of CCL-210 retains a complete GPI-anchor and is therefore insoluble.

CCL-210 CM was partitioned into soluble and insoluble phases using the non-ionic detergent Triton X-114 to assess the solubility of THY1 detected in it. Insoluble
phase and soluble phase were submitted to western blot analysis using K117 as the primary. None of the THY1 detected in the CM of CCL-210 was retained in the soluble phase. Rather, THY1 detected in the CM of either cytokine stimulated or un-stimulated NHLF partitioned exclusively into the insoluble phase [Figure 6. A]. Moreover, the vast majority of THY1 detected in the pre-concentrated CM, retained following centrifugation at 1,200 x g, was removed following centrifugation at ≥ 21,000 x g [Figure 6. B].
DISCUSSION

The monoclonal antibodies from clones K117, 5E10, and AS02 recognize human THY1 at the surface of cells. Thus, these antibodies recognize epitopes displayed by THY1 while in a native conformation. Additionally, all three detect THY1 in western blots, but not under reducing conditions. This suggests, these antibodies recognize epitopes comprised of segments in the polypeptide held in close proximity by disulphide bonds. The mature THY1 polypeptide has four cysteine residues. As a member of the immunoglobulin superfamily, each cysteine is predicted to form a disulfide bond with one of the other under oxidizing conditions [96]. The complete deglycosylation of THY1 does not affect the affinities these antibodies have for it. Therefore, the epitopes are likely confined to a region of the THY1 polypeptide. Taken together, recognition of THY1 by K117, 5E10, and AS02 requires the THY1 polypeptide be in a conformation it assumes at the surface of cell membranes.

Molecular dynamic models comparing rodent Thy1 inserted into a lipid monolayer, vs. delipidated THY1 as if by GPLD1, vs. without any portion of a GPI anchor, demonstrate distinct conformational differences. The conformation of rodent Thy1 without any portion of a GPI anchor is intermediate between the other two but closer to the GPLD1 delipidated model. Interestingly, these same studies suggest the glycan chain of the GPI anchor is tightly folded on itself, bringing the protein in close proximity to the cell surface. A bulk of it may even fit into a lectin-type binding site on the adjacent surface of THY1[90], [98]. The conformational differences are appreciable on the surface of Thy1 opposite the GPI anchor; known epitopes in these areas become compromised [90], consistent with altered antibody affinity. For Thy-1 and GPI-anchored
proteins, the general consensus is that delipidation induces a stable change in conformation that manifests itself in a change in antibody affinity [91]. The positions of TYR residues in human THY1 are better suited than those in rodent Thy1 for using circular dichroism spectra to detect conformational changes. A discernable and stable shift in the circular dichroism spectrum of human THY1 occurs within an hour of its delipidation. Taken all together, antibodies that recognize human THY1 with a GPI anchor are predicted to have lower affinity for THY1 if it is delipidated.

We evaluated the relative affinity of three widely available monoclonal THY1 antibodies, K117, 5E10, and AS02, for the mature THY1 polypeptide with a complete GPI anchor, delipidated by PI-PLC, and expressed as a soluble recombinant by omitting the GPI attachment signal. Of the three, AS02 was shown by another group to detect an increase of THY1 in the supernatant of PI-PLC treated human fibroblast relative to untreated. THY1 was also detected in the supernatant of untreated cells, however [38]. In order to detect the mature THY1 polypeptide independent of conformation, recombinant forms were engineered to be expressed with an N-terminal FLAG tag. Our findings demonstrate that recognition of THY1 by monoclonal antibodies from clones K117, 5E10, and AS02 in western blots is abolished or greatly diminished if THY1 is made soluble by PI-PLC or expressed without a GPI anchor attachment signal. Remarkably, GPI anchors can mediate the conformation of THY1 required for recognition by K117 and 5E10 from as far away as 15 AA and attached by a non-endogenous GPI anchor attachment signal. Moreover, detection of THY1 – GPI(DAF) and – GPI(TR3) suggest that the epitopes these antibodies bind do not encompass both the polypeptide and the glycan core of the GPI anchor.
Recombinant soluble Thy-1 has been designed in a number of different ways. In one design, three AA followed by a stop codon were introduced immediately following CYS 130 of rat Thy1.1 [95]. The additional AA, GGS, were included to allow it to be purified by affinity chromatography using OX-7 [95], shown to lose affinity for Thy1 if delipidated [90]. Though our FLAG – sTHY1 has a 6 AA extension, it was not detected in western blots using K117, 5E10, or AS02 [Figure 3B and Supplementary Figure 1]. Additionally, sTHY1 – 6X HIS, with a 12 AA extension, was not detected in western blots using K117. In a second design, six histidines followed by a stop codon were introduced immediately following LYS 129 of human THY1, thereby omitting CYS 130. Interestingly, this form of recombinant soluble THY1 was detected in western blots by AS02. Also, it was implemented to establish the standard curve in a sandwich ELISA in which AS02 and 5E10 were the detection antibodies [28]. Two potential explanations could account for why these antibodies detect this form but not ours. One, the disulfide bond formed with CYS 130 may place a constraint at the C-terminus that sequesters elements downstream of it. This is supported by reducing conditions exposing the histidine epitope at the C-terminus of sTHY1– 6X HIS in western blots. Two, detection by AS02 and 5E10 may require a greater amount of a soluble THY1 relative to the GPI-anchored form, in which case absolute values for each would not be comparable.

The integrin and syndecan-4 binding motifs RLD and RETKK, respectively, were indentified and characterized using a recombinant hybrid of Thy-1 in which the GPI-attachment signal is replaced by the Fc fragment of human IgG1. Though soluble, the Fc fragment molecular mass at ~25.6kDa is twice the mature THY1 polypeptide. The relatively large size could presumably supply the constraint at the carboxyl terminus for
THY1 antibody recognition. THY1-Fc forms a dimer through the Fc fragments making it further unsuited as a surrogate for WT soluble THY1 [99]. We tested whether commercially available THY1-Fc (ALX-522-091) is recognized by K117. A band just over 100kDa was detected suggesting THY1-Fc has a conformation more akin to GPI anchored THY1 (data not shown). Although without an ability to detect THY1-Fc by western blot independent of its conformation, the possibility can not be discounted that the recognition is non-specific. It is not known whether the binding motifs RLD or RETKK are altered in the soluble THY1 conformation in a manner that would influence affinity for integrins or syndecan-4. Potential affinity differences aside, the bivalent nature of THY1-Fc likely cross links cell surface receptors that native sTHY1 would not. Absent a transmembrane domain, clustering is an important mechanism by which Thy-1 signals [58], [100]. Thus, soluble THY1-Fc most likely better approximates WT THY1 at the cell surface.

There are only two known mammalian enzymes that release GPI-anchored proteins by cutting within the GPI moiety, GPLD1 [29–31] and ACEI [32–34]. Both have been shown to release THY1 from cell surfaces [26], [33], [34]. ACE-I cleaves between the first and second mannose of the GPI moiety leaving ethanolamine-phosphate-mannose [33], [34]. GPLD1 cleaves the inositol phosphate linkage within the GPI moiety so the phosphate is retained by the diacylglycerol [31], [91], [101]. Compared with the remnant GPLD1 leaves behind, ACE-I leaves behind one more similar in size to a small AA extension and less similar to the one PI-PLC generates. However, it is unknown if THY1 released by ACEI is recognized by antibodies demonstrated to be deficient in recognizing other forms of soluble THY1. The GPI anchor remnant, ethanolamine-
phosphate-mannose, could act in a similar manner as the three AA at the C-terminus of sTHY1.1 – GGS.

Despite a difficulty in recognizing soluble THY1, K117 detects basal levels of THY1 in the CM of NHLF and the increase of THY1 that comes with pro-inflammatory cytokine treatment. These incongruous results led to experiments to assess the solubility of THY1 detected in CM. As suggested, THY1 detected in the CM of CCL-210 is entirely insoluble. Differential centrifugation revealed the THY1 to be associated with a rather large particle as it was cleared from the CM at speeds as low as 21,000 × g.

A group recently published detecting elevated THY1 in the cell-free post-digestion supernatant, a by-product in processing prostate tissue for cell sorting by collagenase digestion. The presence of THY1 in the supernatant was attributed to either “cell shedding, secretion, or enzymatic release.” However, this was done by western blot using 5E10 as the primary antibody [39]. Based on the results reported here, the THY1 thus detected likely retains a complete GPI anchor. Mass spectrometry, but not western blot, analysis of urine from patients with prostate cancer identified THY1; conversely, THY1 was not detected in the urine of post-prostatectomy patients [39]. It may be that the THY1 in the urine is soluble.

We report that K117, 5E10, and AS02 are deficient in detecting soluble forms of THY1 by western blot. Thus, there is a need to develop antibodies to THY1 that take into consideration the conformation of delipidated THY1. Additionally, the conformation of recombinant forms of THY1 used as controls in assays that rely on antibody recognition must be evaluated carefully. Beyond antibody recognition, the conformation of recombinant forms of THY1 used to elucidate its function should also be evaluated. The
aforementioned considerations are summarized and depicted in figure 7. Furthermore, the nature of THY1 released by cytokines, which appears to maintain its membrane-bound conformation and may have important biological significance, needs to be evaluated further.
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FIGURE 1. Primary Structures of and N-terminally tagged recombinant THY1

(A) Full length diagrams of primary structures for wild type THY1 and N-terminally tagged recombinant THY1. (B) Alignment of the AA sequences at the N-terminus of N-terminally tagged recombinant THY1 with wild type THY1.
FIGURE 2. Primary Structures of recombinant THY1 with modifications to the C-terminus. (A) Full length diagrams of primary structures for wild type THY1 and recombinant THY1 with modifications to the C-terminus. (B) Alignment of the AA sequences at the C-terminus of recombinant THY1 with modifications to the C-terminus with wild type THY1.
FIGURE 3. Complete GPI anchor of THY1 required for recognition by monoclonal antibodies raised against membrane bound form. (A) CE were collected from NHLF and RFL-6 stably transfected with pcDNA3.1/Zeo(+) (pcDNA) or pcDNA3.1/Zeo (+) with a recombinant form of THY1 cloned in. The recombinant forms included wild type (WT) THY1 (THY1), THY1 – GPI(DAF) (DAF), THY1 – GPI(TR3) (TR3), and FLAG – THY1 (FLAG). Mature THY1 polypeptide was detected in these CE by western blot using the K117 monoclonal antibody as the primary. (B) RFL-6 stably expressing FLAG – THY1 were washed with serum free media then provided either serum free F-12K or 0.1U/mL PI-PLC in serum free F-12K for 24 hrs. RFL-6 stably expressing FLAG – sTHY1 were provided serum free F-12K for >48 hrs. Mature THY1 polypeptide was detected in the CE or CM by western blot using the K117 and AS02 monoclonal antibodies as the primary. After stripping the membranes of antibodies for detection of mature THY1 polypeptide, the FLAG epitope was detected by western blot using the M2 monoclonal antibody as the primary.
FIGURE 4. Monoclonal antibodies from clones K117 and 5E10 recognize epitopes on THY1 independent of its glycosylation, but are abolished under reducing conditions. NHLF extract was diluted in water and brought to 1% SDS with a 10% stock solution then incubated at 100°C for 10 min. 10X G7 reaction buffer and 10% NP40 were added to an aliquot of the solution diluted in water to a final concentration of 1X and 1%, respectively. After adding PNGase F, this reaction mixture and the initial dilution of cell lyate were incubated at 37°C for 1 hour. After this incubation period, the former was diluted with PBS so that each had the same total protein concentration. Then, 5X NR loading buffer was added to each to a final concentration of 1X. Two aliquots were taken from the initial dilution of cell lyate in loading buffer and brought to either 100mM DTT (DTT) or 2.5% β-mercaptoethanol (β) by adding small volumes of concentrated stock solutions. Mature THY1 polypeptide was detected in these samples by western blot using the K117 monoclonal antibody as the primary.
FIGURE 5. Characterization of soluble recombinant THY1 with 6 histidine epitope tag at C-terminus. (A) RFL-6 stably expressing sTHY1 – 6X HIS were provided serum free F-12K for >48 hrs. NR loading buffer was added to CM containing THY1 – 6X HIS and NHLF CE. An aliquot taken from the CM in loading buffer was brought to 2.5% β-mercaptoethanol by adding a small volume of concentrated stock solution. All three preparations were heated for 5 min at 95°C. Two aliquots of the CM in NR loading buffer were run between NHLF CE and the aliquot of CM containing 2.5% β-mercaptoethanol (β). Mature THY1 polypeptide was detected in the CE and CM by western blot using the K117 as the monoclonal antibody as the primary. After stripping the membranes of antibodies for detection of mature THY1 polypeptide, the 6X HIS epitope was detected by western blot. (B) RFL-6 stably expressing sTHY1 – 6X HIS were provided serum free F-12K for >48 hrs. CM was diluted in water and brought to 1% SDS with a 10% stock solution then incubated at 100°C for 10 min. 10X G7 reaction buffer and 10% NP40 were added to an aliquot of the solution diluted in water to a final concentration of 1X and 1%, respectively. After adding PNGase F, this reaction mixture and the initial dilution of cell lyate were incubated at 37°C for 1 hour. After this incubation period, the former was diluted with PBS so that each had the same total protein concentration. The 6X HIS epitope was detected in CM by western blot.
FIGURE 6. THY1 in the CM of NHLFs is insoluble. (A) CCL-210 were washed with serum free MEM then serum starved in serum free MEM for 24 hrs. After serum starving, the cells were washed again and provided either MEM or 20ng/mL TNFα and IL-1β in MEM for 24 to 48 hrs. Using Triton X-114, concentrated CM was partitioned into an aqueous and (AP) insoluble phase (IP). Mature THY1 polypeptide was detected in partitioned and pre-partitioned CM by western blot using the K117 monoclonal antibody as the primary. (B) CCL-210 were washed with serum free MEM then serum starved in serum free MEM for 48 hrs. After serum starving, the cells were washed again and provided MEM for 48 hrs. Un-concentrated CM was submitted to differential centrifugation of 1,200 × g and 21,000 × g then precipitated with methanol. Mature THY1 polypeptide was detected in the precipitated material of total (T) prior to, supernatant (S) after, and pellet (P) after 21,000 × g by western blot using the K117 monoclonal antibody as the primary.
FIGURE 7. Diagram of mature Thy-1 both naturally occurring and artificially generated additions to the C-terminus with potential changes to the affinity with natural ligands and antibodies. The effects most additions to the C-terminus have on the conformation of THY1 with respect to affinity (→) for antibodies have been demonstrated by our and others’ work. Categorizing the conformation of THY1 as “soluble” may be a misnomer. Some forms of recombinant soluble Thy-1 with either a specific AA extension or possibly omission of ceratin AAs, e.g. CYS 130, can restore affinity with antibodies raised against GPI-anchored Thy-1. The conformation assumed by Thy-1 released from cell surfaces with ACEI, with respect to antibody affinity, is not known. Moreover, the effect conformational differences may have on the affinity (→) for known natural ligands or whether new ones are gained is not known. All are important to consider when raising antibodies to, using a recombinant form of, or examining the function of Thy-1.
SUPPLEMENTARY FIGURE 1. Complete GPI anchor of THY1 required for recognition by the monoclonal antibody 5E10. RFL-6 stably expressing FLAG-THY1 were washed with serum free media then provided either serum free F-12K or 0.1U/mL PI-PLC in serum free F-12K for 24 hrs. RFL-6 stably expressing FLAG-sTHY1 were provided serum free F-12K for >48 hrs. Mature THY1 polypeptide was detected in the CE or CM by western blot using the 5E10 monoclonal antibody as the primary. After stripping the membranes of antibodies for detection of mature THY1 polypeptide, the FLAG epitope was detected by western blot using the M2 monoclonal antibody as the primary.
References


CHAPTER 4

CONCLUSIONS

Summary of Results

Four specific aims were conceived to elucidate mechanisms and consequences of Thy-1 shedding. (1) Identify mechanism(s) by which pulmonary fibroblasts shed Thy-1 from their cell surface in response to pro-fibrotic stimuli. (2) Characterize the C-terminus of Thy-1 cleaved from fibroblasts treated with pro-fibrotic stimuli or by increasing endogenous levels of “Sheddases”. (3) Identify residues of Thy-1 that modulate susceptibility to or are required for its shedding from pulmonary fibroblasts elicited by pro-fibrotic stimuli. (4) Measure the concentrations of soluble Thy-1 and of identified sheddases in the BAL fluid of patients with lung fibrosis and determine if a correlation exists between them.

Aims 1 and 2 are addressed in the Chapter 3 “Shedding Paper”. Unexpectedly, THY1 is not made soluble during its release, as was presumed, and associated with a rather large particle [Figure 6 A and B from CHAPTER 3]. Specifically, the “mechanism” is likely the formation of some variety of shedding microvesicle [102], [103] and the C-terminus of THY1 retains a complete GPI-anchor. The monoclonal antibody from clone K117 used to detect THY1 released from pulmonary it deficient in its ability to detect both enzymatic generated or recombinant expressed soluble THY1. This was the impetus to evaluate the solubility of THY1 released from pulmonary fibroblasts and the relative affinity for soluble THY1 of other monoclonal antibodies
raised against GPI-anchored THY1. To do so, we engineered recombinant FLAG–THY1 and FLAG–sTHY1 and are the first to express human THY1 with an epitope tag at the N-terminus of the mature protein. Specific aim 3 was predicated on identifying an enzyme that releases THY1 from the cell surface by separating it from the lipid moieties of the GPI anchor. Anticipating an enzyme that sheds THY1 might require disruption of lipid rafts to function, we endeavored to produce recombinant forms of THY1 that while still GPI-anchored would localize to non-native membrane micro-domains. Though no enzyme was identified, recombinant THY1 hybrids were engineered to have GPI anchors attach using the DAF and TRAIL-R3 GPI-attachment signals, designated THY1–GPI(DAF) and THY1–GPI(TR3). Both were repurposed to show that GPI anchors can mediate the conformation of THY1 required for recognition by K117 and 5E10 from as far away as 15 AA and attached by a non-endogenous GPI anchor attachment signal. As the results within Chapter 3 “Shedding Paper” make clear, measuring the concentration of THY1 employing antibodies raised against GPI-anchored THY1 is likely to be problematic. A predilection for a particular conformation would make measurements for the absolute values of GPI-anchored and soluble THY1 non-comparable. In this chapter, the future directions of the four original specific aims are discussed.
Specific Aim One: Identify mechanism(s) by which pulmonary fibroblasts shed Thy-1 from their cell surface in response to pro-fibrotic stimuli.

Knowing THY1 detected in the CM of NHLF to be in-soluble and associated with a large particle, the next step will be to characterize the particle THY1 associates with. Extracellular membrane vesicles (MVs) range from 30-1000nm in diameter [102], [103], and demonstrated to arise from lipid rafts [104]. Thus, MVs are a likely candidate for the THY1 containing particle. Moreover, MVs carry “cargo”, e.g. RNAs, lipids, and proteins, which can be transmitted to other cells [102]. Should the large THY1 containing particle be a MV, then characterizing it will entail measuring the size and assessing the “cargo”.

Localization of THY1 to its native membrane micro domain is required for its signaling [58], [105] but is also thought to afford THY1 on endothelial cells as well as on circulating T-cells protection from GPLD1 which is abundant in serum [26]. The same protection is afforded to THY1 against ACEI, the enzyme most recently demonstrated to have GPI specific lipase activity [33]. Currently, there is no known in-vivo mechanism for THY1 release by GPLD1 and ACEI that lipid raft disruption approximates. An expansion of specific aim (1) will be to determine if THY1 on MVs is protected from release by GPLD1 and ACEI as it is at the surface of cell membranes.

Specific Aim Two: Characterize the C-terminus of Thy-1 cleaved from fibroblasts treated with pro-fibrotic stimuli or by increasing endogenous levels of “Sheddases”.

This specific aim has expanded into characterizing the conformation of recombinant and natural forms of THY1 with respect to antibody recognition and
function. The utility of placing an epitope tag at the N-terminus of mature forms of THY1 is it allows them to be quantified independent of their conformation. Several recombinant and naturally occurring forms of soluble THY1 remain to be evaluated with respect to recognition by monoclonal antibodies from clones K117, 5E10, and AS02. Specifically, THY1 – Fc, sTHY1 – GSS, and ACE delipidated THY1 should be evaluated in western blots as was done in CHAPTER 3 with a FLAG tag at the N-terminus of the mature proteins. Moreover, delipidated or recombinant soluble THY1 having a conformation akin to delipidated should be evaluated in functional assays to determine whether the binding properties associated with membrane bound form are the same. This includes the possibility of divergent ligands for each.

CD52 is another GPI-anchored protein that antibody recognition suggests has an appreciably different conformation when delipidated. Delipidation of CD52 with PI-PLC renders it unrecognizable by CAPATH-1H even if the 2nd position of the myo-inositol ring retains a palmitoyl residue [106]. Similar to THY1, CD52 antibodies can bind to deglycosylated, CD52 but not to the peptide alone. Additionally, a two AA extension of the CD52 peptide restores reactivity [107]. With regard to function, both membrane-bound and soluble CD52 have been purified in association with a fragment of semenogelin-I, suggesting a similar conformation in the semenogelin-I binding site. Given the small size of the CD52 polypeptide, only 12 AAs [108], the epitopes of CD52 antibodies eliminated with delipidatation and binding site for semenogelin-I are very likely to overlap. To allow for this, a remnant of the GPI anchor may approximate the two AA extension that restored reactivity to synthetic CD52. ACE-I cleaves between the first and second mannose of the GPI moiety leaving ethanolamine-phosphate-mannose
GPLD1 cleaves the inositol phosphate linkage within the GPI moiety so the phosphate is retained by the diacylglycerol [101]. Compared with the remnant GPLD1 leaves behind, ACE-I leaves behind one more similar to the two AA extension and less similar to the one PI-PLC generates. This has yet to be experimentally determined.

**Specific Aim Three: Identify residues of Thy-1 that modulate susceptibility to or are required for its shedding from pulmonary fibroblasts elicited by pro-fibrotic stimuli.**

GPI-anchors, despite their name, do not simply anchor polypeptide ecto-domains to the cell surface. Rather, they serve the ecto-domain as a temporal [109] and spatial mediator [58], [105] of signal transduction, traffic it to either an apical or basal lateral surface [110], [111], and in some instances even confer a specific conformation [112]. These individual effects are likely interdependent or at least act synergistically to support the function of GPI-anchored proteins.

Classic methods for characterizing lipid raft domains concurrent with proteins that reside within them employ their relative insolubility in solutions of Triton X-100 at 4°C. The resulting detergent-insoluble glycolipid-enriched membranes are buoyant and when subjected to density gradient centrifugation float in the low density fractions along with associated proteins. Depending on the density gradient and number of fractions taken off it, distinctive partitioning profiles can be generated for different proteins [113]. The partitioning profiles of the DAF and TRAIL-R3 eGFP hybrids are very distinctive from one another and identical to the native proteins from which the GPI-attachment signals originate. Remarkably, these profiles remained the same regardless as to how the hybrid proteins came to reside at the cell surface whether do to transfecting in an expression
plasmid or having them “painted on” [27]. Unlike THY1, not all of DAF at the cell surface is protected from being shed by ACEI [33]. The partitioning profiles of THY1, THY1 – GPI(DAF), and THY1 – GPI(TR3) have been determined as described in Materials and Methods. THY1 and THY1 – GPI(DAF) had very similar profiles while THY1 – GPI(TR3) was only detected in the first fraction [Figure 1]. An interesting observation made while maintaining the cell lines stably expressing these proteins was the band intensity was always less for THY1 – GPI(TR3) in western blots of equal total protein relative to the others using K117 as the primary [ Figure 3A from CHAPTER 3]. The cell surface expression of THY1 – GPI(TR3) was also less as determined by flow cytometry (data not shown). There exists the possibility that heterogeneity in the GPI anchors of these 3 produce subtle conformational differences between them. Additionally, THY1 – GPI(TR3) may be demonstrating the initial purpose of its design, e.g. making THY1 more susceptible to shedding. As with WT THY1, neither hybrid is detectable in the CM of RFL-6 stably expressing them treated with PI-PLC (data not shown). To explore these possibilities these hybrids should be re-engineered to express with a FLAG epitope as FLAG – THY1 does. Should either be more susceptible to shedding during routine cell culture or in designed experiments, this will allow the released soluble form to be detected.
Specific Aim Four: Measure the concentrations of soluble Thy-1 and of identified sheddases in the BAL fluid of patients with lung fibrosis and determine if a correlation exists between them.

For THY1 and GPI-anchored proteins, the general consensus is delipidation induces a stable change in conformation that manifests itself in a change in antibody affinity [91]. Certain antibodies have greater affinity for the delipidated conformation as the clone TS2/9 has for PI-PLC released CD58 [37]. The first description of a GPI-anchor was of the one attached to Variant Surface Glycoprotein (VSG) from the parasite T. brucei [114]. Several monoclonal antibodies to VSG that recognize it at the cell surface were shown to lose their affinity with delipidation [91], [115]. VSG on the cell surface of living Trypanosomes frequently do not react with the same VSG on immunoblots and thought to occur because the protein recovered from cell lysates to raise the antibodies was delipidated by endogenous GPI-PLC [115]. Taken together, it seems likely that inoculating an animal with delipidated or a recombinant THY1 shown to have a conformation unrecognized by K117, 5E10, and AS02 would raise antibodies better suited for applications involving soluble THY1. The recombinant forms of THY1 discussed allow such antibodies to be easily evaluated in this respect.

Beyond the difference in conformations of GPI-anchored and soluble THY1 is the possibility that heterogeneity in the GPI anchor [116] may induce conformations that go undetected. The Folate Receptor (FR) expressed in two variant sublines of murine L1210 leukemia cells was shown to differ in relative affinity for its ligands. This difference could not be attributed to differences in AA sequence, as they were identical, nor did partial deglycosylation with N-glycanase modify ligand binding characteristics of either. The difference in ligand affinity between the two sources was attributed to differences in
their GPI anchors. Specifically, the FR with greater ligand affinity was demonstrated to be acylated in the inositol ring [112]. Thus, there may be a need to develop a knock in mouse that expresses N-terminally epitope tagged mature murine Thy1, e.g. FLAG – Thy1.2, under the control of the endogenous Thy1 transcriptional regulatory elements. Such a mouse could reveal previously undetected membrane bound Thy1 expression on cells and soluble Thy1 in bodily fluids.
Materials and Methods

Partitioning profiles of THY1, THY1 – GPI(DAF), and THY1 – GPI(TR3) formed by sucrose gradient ultracentrifugation.

RFL-6 stably expressing THY1, THY1 – GPI(DAF), and THY1 – GPI(TR3) were lysed in 1X TNE buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, and 5mM EDTA) containing 0.1% Triton X-100 (Sigma-Aldrich) at 4°C. Cell lysates containing buoyant detergent-insoluble glycolipid-enriched membranes were brought to 35% OptiPrep by adding 60% OptiPrep (Sigma-Aldrich D1556) containing 0.1% Triton X-100. 30% OptiPrep in 1X TNE was layered on, followed by a small volume of 1X TNE. The resulting sucrose density gradient was submitted to ultracentrifugation of 45,000rpm for 4 hrs at 4°C. Afterwards, 7 fractions of equal volume were taken off the top. Fractions were loaded in an acrylamide gel in the order they were collected from left to right. The mature THY1 polypeptide was detected in these fractions by western blot using the K117 monoclonal antibody as the primary as described in CHAPTER 3.
Figure 1. Partitioning profiles of THY1, THY1 – GPI(DAF), and THY1 – GPI(TR3) formed by sucrose gradient ultracentrifugation. RFL-6 stably expressing THY1, THY1 – GPI(DAF), and THY1 – GPI(TR3) were lysed in 1X TNE buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, and 5mM EDTA) containing 0.1% Triton X-100 (Sigma-Aldrich) at 4°C. Cell lysates containing buoyant detergent-insoluble glycolipid-enriched membranes were brought to 35% OptiPrep by adding 60% OptiPrep (Sigma-Aldrich D1556) containing 0.1% Triton X-100. 30% OptiPrep in 1X TNE was layered on, followed by a small volume of 1X TNE. The resulting sucrose density gradient was subjected to ultracentrifugation of 45,000rpm for 4 hrs at 4°C. Afterwards, 7 fractions of equal volume were taken off the top. Fractions were loaded in an acrylamide gel in the order they were collected from left to right. The mature THY1 polypeptide was detected in these fractions by western blot using the K117 monoclonal antibody as the primary.
General References


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