# Chapter 1
# Thy-1 Modulates Neurological Cell–Cell and Cell–Matrix Interactions Through Multiple Molecular Interactions

**Lisette Leyton and James S. Hagood**

**Abstract** Thy-1, or CD90, is a glycosylphosphatidylinositol-linked cell surface glycoprotein expressed on multiple cell types, including neurons, thymocytes, fibroblasts, endothelial cells, mesangial cells, and some hematopoietic and stromal stem cells. Thy-1 is developmentally regulated and evolutionarily conserved. Its cellular effects vary between and in some cases within cell types, tissues, and species, indicating that its biological role is context dependent. However, it most often seems to affect cell–cell or cell–matrix interactions and cellular adhesion and migration. In the nervous system, Thy-1 mediates bidirectional cell–cell communication, which modulates cell–matrix adhesion. Neurons express high levels of Thy-1, which interacts with $\alpha_v\beta_3$ integrin present in astrocytes and stimulates increased astrocyte adhesion to the underlying surface (*trans* signaling) and in neurites, the same ligand–receptor association triggers neurite retraction and inhibition of axonal growth (*cis* signaling). Although Thy-1 lacks a cytoplasmic domain, it affects multiple intracellular signaling cascades through interaction with a number of molecules within lipid raft microdomains. Improved understanding of how this enigmatic adhesion molecule modulates signaling and cell phenotype may yield novel insights into neurodevelopment and nerve recovery after injury.

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## 1.1 Introduction

Thy-1 (thymocyte differentiation antigen 1), also known as CD90 (cluster of differentiation 90), is a highly conserved cell surface molecule that can exist in membrane-bound and soluble forms. Thy-1 is developmentally regulated and expressed in specific cell types, including neurons, retinal ganglion cells, subsets of fibroblasts, vascular pericytes, activated endothelial cells, mesangial cells, and hematopoietic and mesenchymal stem cells. Previous reviews have focused on its immunologic and non-immunologic roles, mechanisms, and consequences of Thy-1-associated signaling and regulation of its expression (Haeryfar and Hoskin 2004; Rege and Hagood 2006a, b; Barker and Hagood 2009a; Bradley et al. 2009). Here, we will consider the role of Thy-1 as an adhesion molecule, mainly in the context of neurobiology.

Thy-1, originally designated as theta ($\theta$) antigen, was initially defined as a leukemia-specific antigen in mice (Reif and Allen 1964; Schlesinger and Yron 1969). There are important species-specific differences in expression (see Sect. 1.4). Thy-1 regulation and signaling have been implicated in several disease states including neuronal injury (Leyton et al. 2001; Schlamp et al. 2001; Barker and Hagood 2009b), pulmonary fibrosis (Rege and Hagood 2006a; Sanders et al. 2007, 2008), some cancers (Abeysinghe et al. 2003; Lung et al. 2005; Fiegel et al. 2008), Graves' disease ophthalmopathy (Khoo et al. 2008), and glomerulonephritis (Minto et al. 2003).

## 1.2 Role of Thy-1 in Neurobiology

Thy-1 is either absent from or expressed in a restricted manner in neurons during development (Xue et al. 1990), but accounts for 2.5–7.5 % of total protein on axon membranes of mature rat neurons (Beech et al. 1983). Thy-1 has been associated with the resolution of neuronal injury. Nerve injury in animal models is associated with decreased Thy-1 expression, with recovery of expression associated with a partial or complete return to the normal or proper physiological neuronal function (Chen et al. 2005). Thy-1 expression in the nervous system is predominantly neuronal, but some human glial cells also express Thy-1, especially following differentiation (Kemshead et al. 1982). Thy-1 inhibits neurite outgrowth on astrocytes, in neurons transfected with either human Thy-1 or mouse Thy-1.2 (Tiveron et al. 1992). Neurite outgrowth is restored in these studies by antibodies against Thy-1, or by addition of soluble Thy-1, suggesting that blocking Thy-1's interaction with a ligand on astrocytes removes the inhibitory effect of Thy-1 on neurite extension (Tiveron et al. 1992). The inhibitory effect of Thy-1 on neurite outgrowth requires its correct localization to native membrane microdomains (Tiveron et al. 1994). Recent epitope mapping studies have characterized the antibody-binding sites that affect neurite extension and found that they recognize not only amino acid sequences, but also the three-dimensional immunoglobulin-like domains and integrin-binding regions (Kuroiwa et al. 2012).

Remarkably, the phenotype of Thy-1 null mice seems to lack significant functional abnormalities involving the nervous system. The principal abnormalities described thus far are inhibition of long-term potentiation in the hippocampal dentate gyrus, inability to transmit social cues regarding food selection, and impaired cutaneous immune responses (Mayeux-Portas et al. 2000; Nosten-Bertrand et al. 1996; Beissert et al. 1998).

In cultures of dorsal root ganglion neurons, interfering with Thy-1 molecular interactions causes the neurons to grow complex processes on the culture substrates. Signaling pathways leading to neurite outgrowth in this case include the activation of both protein kinase A (PKA) and Src, which affect the activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase/cAMP response element-binding protein (MEK/Erk/CREB) pathway, although a direct link between Thy-1 engagement by antibodies and these signaling cascades has not been confirmed (Chen et al. 2007; Yang et al. 2008). Improved understanding of the molecular mechanisms involved in Thy-1-mediated neurite outgrowth inhibition may help in designing interventions to block the negative effects of Thy-1 on the repair of neuronal processes.

More recently, the ligand of Thy-1 present in mature astrocytes has been revealed. An $\alpha_v\beta_3$ integrin has been reported to bind to Thy-1 and trigger clustering of Thy-1, inactivation of Src, and neurite outgrowth inhibition (Herrera-Molina et al. 2012). The complete flow of signaling events initiated as a consequence of Thy-1 integrin interactions awaits further investigation.

## 1.3 Thy-1 in Non-neuronal Contexts

The functions of Thy-1 in immunity and inflammation, as well as in regulation of cell adhesion and migration, have been reviewed previously (Bradley et al. 2009; Haeryfar and Hoskin 2004; Rege and Hagood 2006b). Additionally, Thy-1 appears to function as a tumor suppressor in several malignancies, including nasopharyngeal and ovarian cancer (Lung et al. 2005). Loss of heterozygosity (LOH) at 11q23.3–q24.3, where *THY1* is mapped in humans, is associated with poor prognosis for ovarian cancer (Cao et al. 2001; Williams and Gagnon 1982). Forced Thy-1 expression suppresses tumorigenicity in the ovarian cancer cell line SKOV-3 (Cao et al. 2001; Abeysinghe et al. 2003). In neuroblastoma, Thy-1 expression correlates inversely with patient survival (Fiegel et al. 2008).

In fibroblast cells, Thy-1 has significant effects on the cell phenotype depending on the tissue origin and context. In lung fibroblasts, Thy-1 suppresses myofibroblastic differentiation and cell migration through effects on Src-family kinases (SFK) and phosphatidylinositol 3-kinase (PI3K) signaling (Barker et al. 2004a; Rege et al. 2006; Sanders et al. 2007). Thy-1, via interaction with $\alpha_v\beta_5$ integrin, inhibits activation of latent transforming growth factor-beta1 (TGF-β1) and myofibroblastic differentiation in lung fibroblasts (Zhou et al. 2010). Conversely, in Graves' disease ophthalmopathy, Thy-1 (+) orbital fibroblasts differentiate into myofibroblasts, while Thy-1 (−) are incapable of doing so, but can differentiate into mature

adipocytes (Koumas et al. 2003). However, more recent evidence suggests that Thy-1 (+) fibroblasts can differentiate into lipofibroblasts upon treatment with a PPARγ ligand, but that they would secrete an as-yet unidentified soluble factor that inhibits such differentiation (Khoo et al. 2008; Lehmann et al. 2010). Thy-1 increases PPARγ, fatty acid uptake, and lipofibroblastic differentiation in fetal lung fibroblasts (Varisco et al. 2012).

## 1.4 Thy-1 Species Differences and Structural Evolution

Thy-1 is an evolutionarily conserved member of the immunoglobulin superfamily (IgSF) (Chen et al. 2005), with significant homology among tunicates, birds, fish, amphibians, rodents, and humans. Among different species, tissue and cellular distribution of Thy-1 expression varies. Mice express Thy-1 on thymocytes, T-lymphocytes, bone marrow stem cells, neurons, and some fibroblasts. In humans, Thy-1 is expressed on a subset of CD34+ bone marrow cells, on a subset of CD34+ and CD3+/CD4+ lymphocytes, and on hematopoietic cells derived from umbilical cord blood and fetal liver, but is absent from mature T cells. In humans, the highest expression levels are on thymic stromal cells (especially fetal) and most fibroblasts. Thy-1 is also expressed in endothelial cells, smooth muscle cells, and some leukemic and lymphoblastoid cells (Feng and Wang 1988). Thy-1 is expressed in neural tissue of all mammalian species studied. In the human nervous system, Thy-1 is expressed primarily in gray matter and in some peripheral nerve fibers (McKenzie and Fabre 1981). Thy-1 is both spatially and temporally regulated during nervous system development; brain expression levels rise nearly 100-fold during early postnatal development (Morris 1985).

Because Thy-1 functions in both the immune system and the nervous system, it may represent a primordial domain of the IgSF ancestry (Cao et al. 2001). Most studies of gene regulation and structure of Thy-1 have been done in the mouse. Murine *thy1* has two alleles which map to chromosome 9, coding for proteins designated Thy-1.1 and Thy-1.2, which are characterized by either arginine or glutamine at position 89. Human *THY1* has no described allelic variants. It is expressed as a 161 aa pro form with a 19 aa signal peptide, which is removed after targeting Thy-1 to the cell membrane (Williams and Gagnon 1982). Thy-1 is variably N-glycosylated, with differing glycosylation among different tissues (Seki et al. 1985; Almqvist and Carlsson 1988; Barclay et al. 1976; Hoessli et al. 1980). Carbohydrate content makes up a third or more of the mass of Thy-1, which ranges from 25 to 37 kDa (Almqvist and Carlsson 1988; Haeryfar and Hoskin 2004). Following cleavage of the C-terminal transmembrane domain, a glycosylphosphatidylinositol (GPI) anchor composed of two fatty-acyl groups is added at residue 131, so that mature Thy-1 is tethered to the outer leaflet of the cell membrane and targeted to lipid rafts (Seki et al. 1985).

The carbohydrate composition of Thy-1 is also developmentally regulated and varies between and within tissues. For example, in rats, sialic acid is much more prominent in thymic Thy-1 than in brain Thy-1, and galactosamine is restricted to brain Thy-1 (Haeryfar and Hoskin 2004).

## 1.5 Thy-1 Regulation

Unusual regulatory elements define the unique expression profile of Thy-1. The Thy-1 promoter is found in an area of high G/C content and lacks a TATA box; it contains two elements traditionally attributed to "housekeeping" genes (Giguere et al. 1985; Spanopoulou et al. 1991). Replacement of the Thy-1 promoter with a heterologous promoter does not abolish the tissue-specific or developmental expression profile (Vidal et al. 1990). Thy-1 expression in the mouse thymus and brain relies on specific sequences in intron 3 and at the 3′ end of intron 1, respectively. Deletion of intron 1 eliminates brain expression while leaving thymic expression intact (Spanopoulou et al. 1988). Interaction of transcription factors with elements within the third intron varies among species (Tokugawa et al. 1997).

A murine *thy1.2* expression cassette has been designed to drive nervous system expression. This cassette is void of all Thy-1.2 coding sequences and the thymic enhancer in intron 3, while retaining the neural enhancer element in the first intron (Campsall et al. 2002).

Thy-1 functions as a tumor suppressor in nasopharyngeal cancer and is downregulated in some tumors by methylation of its promoter (Lung et al. 2005). In human and rat lung fibroblasts, CpG (cytosine-guanine) islands in the Thy-1 promoter are hypermethylated in the Thy-1-negative fibroblast subpopulation, but not in the positive. A DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, restores Thy-1 expression in Thy-1 (−) fibroblasts (Sanders et al. 2008). Trichostatin A (TSA, a histone deacetylase inhibitor) also restores Thy-1 expression in Thy-1 (−) cells associated with depletion of trimethylated H3K27, enrichment of trimethylated H3K4 and acetylated H4, and demethylation of previously hypermethylated CpG sites, indicating interaction of the DNA methylation and histone modification systems in cell-specific epigenetic silencing of Thy-1 (Sanders et al. 2011).

Posttranscriptional regulation of Thy-1 mRNA also influences the temporal and spatial expression of Thy-1 protein in developing mouse nervous system, though the exact mechanisms are still not well characterized (Xue and Morris 1992). Heterokaryons generated from fusion of mature Thy-1.1-expressing neurons with immature Thy-1.2-negative neurons become Thy-1 negative within 16 h of fusion. However, Thy-1.2 expression becomes evident within 3–4 days in culture coincident with re-expression of Thy-1.1. The initial inhibition of Thy-1.1 expression was concluded to be the consequence of a developmentally regulated diffusible suppressor molecule (Saleh and Bartlett 1989). This lends support to developmental regulation of Thy-1 in the nervous system being, at least in part, a posttranscriptional event.

Soluble Thy-1 has been detected in serum, cerebrospinal fluid (CSF), wound fluid from skin ulcers, and synovial fluid from rheumatoid arthritis (Almqvist and Carlsson 1988; Saalbach et al. 1999). Possible methods for production of soluble Thy-1 include alternative mRNA splicing, omitting addition of the GPI anchor, or enzymatic cleavage of Thy-1 from the cell surface. Interestingly, the soluble Thy-1 detected in CSF has slightly higher MW than cellular Thy-1 in the cerebral cortex,

attributed to unique glycosylation patterns and suggesting that soluble Thy-1 in CSF could originate from a region of the brain other than the cerebral cortex. The significance and origin of soluble Thy-1 in CSF are unclear. The susceptibility of Thy-1 to cleavage by phospholipases varies from one cell type to another (Naquet et al. 1989). Localization of Thy-1 to cholesterol-rich lipid rafts is thought to protect it from GPI-PLD present in serum (Bergman and Carlsson 1994). Release of Thy-1 could also result from proteolysis. The exact mechanism(s) of Thy-1 shedding and possible roles of shedding in normal biology and in disease have yet to be determined. It is important to note that Thy-1 lacking the GPI anchor very often becomes unrecognizable by antibodies against the membranous form (Kukulansky et al. 1999). In the human uterine cervix, vascular pericytes expressing Thy-1 appear to secrete Thy-1 (+) vesicles, which communicate with basal epithelial cells (Bukovsky et al. 2001). The biological significance of this intriguing phenomenon is uncertain.

## 1.6 Thy-1 and Non-neuronal Cell Adhesion Signaling

Focal adhesion assembly/disassembly and additional cell–cell and cell–matrix interactions are generally regulated by integrin signaling. Thy-1–integrin interactions appear to regulate a number of heterotypic interactions between cells. Thy-1 expressed on endothelial cells interacts with $\beta_2$ and $\beta_3$ integrins on leukocytes and with melanoma cells (Choi et al. 2005; Wetzel et al. 2004; Avalos et al. 2002; Saalbach et al. 2000, 2002, 2005). Thy-1 on endothelial cells interacts with $\alpha_v\beta_3$ on melanoma cells and with leukocyte $\alpha_X\beta_2$ and $\alpha_M\beta_2$ (Wetzel et al. 2004; Saalbach et al. 2000, 2002, 2005; Choi et al. 2005), regulating melanoma and leukocyte *trans*-endothelial migration in vitro (Saalbach et al. 2005; Wetzel et al. 2004). It is unknown whether Thy-1 null mice have abnormal leukocyte recruitment or resistance to melanoma metastases.

In Thy-1 (−) fibroblasts, SFK and p190 Rho GTPase-activating protein (GAP) activation results in inactive RhoA, promoting focal adhesion (FA) disassembly; Thy-1 expression decreases SFK and p190 RhoGAP activation, which activates Rho and promotes FA formation (Barker et al. 2004a). FA are supramolecular complexes containing structural proteins, signaling molecules, and adapter proteins that include talin, vinculin, α-actinin, focal adhesion kinase (FAK), SFK, p130CAS, paxillin, and tensin, which mediate cell adhesion to the extracellular matrix (ECM) [reviewed in Burridge and Chrzanowska-Wodnicka (1996), Lawson and Schlaepfer (2012), Zamir and Geiger (2001)].

Thy-1 expression inhibits fibroblast migration in vitro (Barker et al. 2004a), likely due to enhanced adhesion of cells to the ECM. Thrombospondin-1 promotes FA disassembly and induces migration only in Thy-1 (+) lung fibroblasts (Rege et al. 2006). This differential response may be important in regulating cell migration in response to factors present during early wound healing following injury; absence of Thy-1 expression, such as in a fibrotic condition or tumor, may promote dysregulated cell migration.

Because the GPI anchor of Thy-1 does not cross the cell membrane, it is unclear how Thy-1 activates intracellular kinases. It is possible that Thy-1 GPI interacts directly with palmitoylated and myristoylated cysteines on these kinases, as has been suggested by Kusumi et al. (2004). Upon aggregation of a critical number of GPI-anchored proteins in rafts of the outer leaflet of the cell membrane, lipid rafts present in the inner leaflet and associated proteins are recruited underneath the outer leaflet of the bilayer (Kusumi et al. 2004). In this manner, communication of two proteins that face opposite sides of the cell membrane can associate functionally. A different possibility is that a third component of the protein complexes exists and that the GPI-anchored protein interacts with intracellular kinases through transducers as has been demonstrated for other GPI-anchored proteins, such as CD55 and CD59 (Shenoy-Scaria et al. 1993). Thus, Thy-1 may interact with cytoplasmic kinases through an associated transmembrane protein. Thy-1-CD3 co-activation engages the lipid raft transmembrane adapter protein linker for activation of T cells (LAT) (Leyton et al. 1999). Thy-1 is known to interact with an 85–90 kDa transmembrane phosphorylated protein possessing binding sites for SH2 domain-containing proteins, including Fyn, Csk, PI3K, Ras GAP, Vav, and Lck (Durrheim et al. 2001). Recently, Thy-1 has been shown to interact with other cell surface and membrane-spanning proteins. Endothelial Thy-1 binds to the adhesion G-protein-coupled receptor CD97 on leukocytes (Wandel et al. 2012). Therefore, the possibility that Thy-1 signals to the cell interior via the formation of a complex containing a transmembrane protein with affinity for both Thy-1 and SFK is a likely model.

## 1.7 Neuronal Thy-1 and Its Interactions with Astrocytes

Astrocytes constitute the most abundant cell population of the brain; they are not merely associated with neurons serving a supportive function, but also establish contacts with the endothelial cells of capillaries and are interconnected through gap junctions, facilitating the communication of other cells with neurons [reviewed in Benarroch (2005)]. Astrocytes can modulate neuronal excitability and synaptic transmission [reviewed in Perea and Araque (2002) and Fellin and Carmignoto (2004)]. In addition, Leyton et al. have reported a bidirectional signaling between neurons and astrocytes. These findings confirmed that astrocytes are much more dynamic components of the central nervous system than originally thought [reviewed in Hansson and Ronnback (2003) and Volterra and Meldolesi (2005)].

The first identified ligand/receptor for Thy-1 was $\beta_3$-containing integrin (Leyton et al. 2001). Stimulation of a rat astrocyte cell line with recombinant Thy-1 in vitro causes morphological changes of astrocytes; their fine-branched processes retract, transforming these cells into a fibroblast-type shape. These events involve a number of cytoskeletal changes, establishing a higher number of adhesive sites at the tips of microfilament bundles that extend to the central region of the cells, generating tension and cell contraction. These events were subsequently demonstrated to be caused by $\alpha_v\beta_3$ integrin in astrocytes (Hermosilla et al. 2008).

On the other hand, the integrin–Thy-1 interactions also induce changes in neurons. HEK293-generated $\alpha_v\beta_3$-Fc integrin has been shown to inhibit the growth of neuronal processes and cause the retraction of existing neurites (Herrera-Molina et al. 2012). Thus, neuron–astrocyte interactions mediated by $\alpha_v\beta_3$ integrin and Thy-1 might represent an important form of bidirectional communication in the nervous system that generates signals in both neurons and astrocytes.

Integrins are surface receptors known to mediate the formation of FA formed at the points of interaction of integrin with the ECM proteins. These FA connect the ECM to the cytoskeleton, which facilitates bundling of actin microfilaments to generate stress fibers (SF) and increase cellular tension (Dubash et al. 2009).

Interaction between Thy-1 and $\alpha_v\beta_3$ integrin present in astrocytes promotes (1) tyrosine phosphorylation of proteins present in the FA such as FAK and p130Cas; (2) recruitment of vinculin, paxillin, and FAK to FA; and (3) formation of FA, adhesion, and spreading of astrocytes over a substratum. The formation of FA and SF in astrocytes stimulated with recombinant Thy-1-Fc occurs through the aggregation of $\alpha_v\beta_3$ integrins and activation of PKCα, the small GTPase RhoA, and its effector Rho kinase (ROCK) (Avalos et al. 2002, 2004, 2009).

Fibroblasts adhering to fibronectin substrate via interactions mediated by the $\alpha_5\beta_1$ integrin receptor decrease the activity of Rho to lower the degree of contractility of cells in suspension, thus allowing spreading on fibronectin within 30 min (Arthur and Burridge 2001). The cooperative interaction of fibronectin with integrins and the proteoglycan Syndecan-4, through its RGD domain and heparin-binding domain (HBD), respectively, leads to the activation of RhoA, increasing the formation of FA and SF, thereby strengthening cell adhesion (Couchman and Woods 1999).

In astrocytes, the interaction of Thy-1 with both integrin and Syndecan-4 is required for adhesion and spreading to produce morphological changes in these cells (Avalos et al. 2009; Leyton et al. 2001). Thy-1 sequence possesses an RGD-like tripeptide shown to be the integrin-binding region. Thy-1 mutated in the third amino acid (aa) of the RLD tripeptide to RLE neither binds the $\alpha_v\beta_3$ integrin nor induces formation of FA (Hermosilla et al. 2008); similarly, mutation of the basic stretch of aa, REKRK, in the Thy-1 molecule, to AEAAA, renders the protein unable to bind heparin, indicating that this region is the HBD of Thy-1 (Avalos et al. 2009; Leyton et al. 2001). Moreover, addition of heparin as a competitive inhibitor of Syndecan-4 binding to Thy-1 causes a decrease in the activation of RhoA in astrocytes and the pretreatment of astrocytes with heparitinase, which digests heparan sulfate proteoglycans and reduces the formation of Thy-1-induced FA and SF. Enhanced cell adhesion and the activation of Rho are inhibited also by overexpression of a dominant negative form of Syndecan-4. Altogether, these results suggest that the formation of FA and SF in astrocytes requires Thy-1 interaction with both integrin and Syndecan-4, through its RLD and REKRK domains, respectively [Fig. 1.1; Avalos et al. (2009)].

Thy-1-induced RhoA-GTP formation induces activation of PKCα (Avalos et al. 2009). PKCα may be activated in response to the aggregation of integrins (Erb et al. 2001; Vossmeyer et al. 2002) or in response to signaling downstream of Syndecan-4 (Dovas et al. 2006). The involvement of PKCα in the activation of RhoA-ROCK in

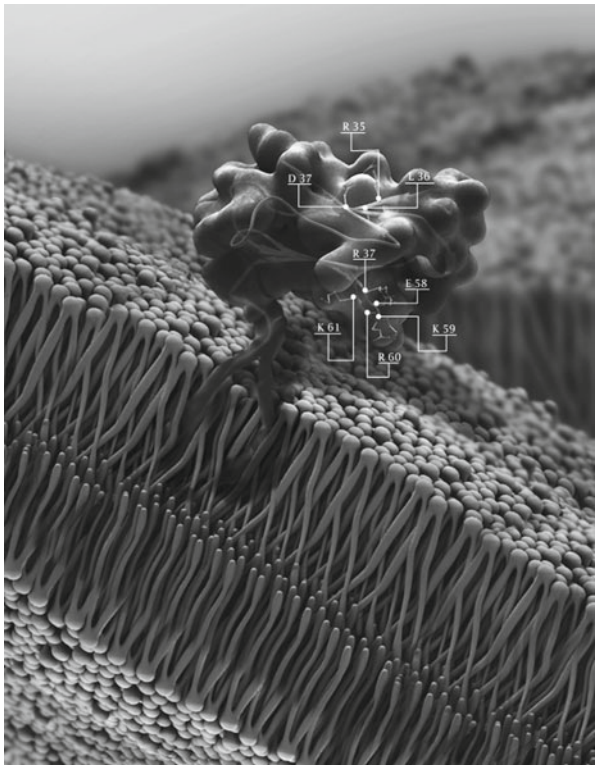


**Fig. 1.1** The conserved RLD tripeptide known to interact with $\alpha_v\beta_3$ integrin and the heparin-binding domain (REKRK) of Thy-1, which binds to Syndecan-4, are indicated. Thy-1 is shown inserted in the outer leaflet of the plasma membrane via a lipid anchor. The primary sequence of amino acids 27–161 of human Thy-1 was used to generate a three-dimensional model (Accession number: AAA61180.1). The PBD generated was used to build the Thy-1 molecule using Autodesk Maya mMaya v.1 Molecular Maya toolkit. Graphics and final images were obtained with Adobe Illustrator and Photoshop (Walter Waymann, Designer)

astrocytes has been demonstrated using the inhibitor of classical PKCs (α, β, and γ) Gö6976 and expressing a dominant negative form of PKCα in astrocytes. Activation of RhoA and the formation of FA and SF decrease in both cases (Avalos et al. 2009). These results indicate that the formation of FA and SF in astrocytes stimulated with Thy-1 depends on PKCα. However, whether PKCα is activated downstream of integrin and/or Syndecan-4 remains unresolved.

Due to the fact that PKCα is a calcium/diacylglycerol-activated kinase, these findings opened new questions that are interesting to explore, e.g., Is the release of $Ca^{2+}$ from the endoplasmic reticulum, enough to activate and maintain PKCα at the membrane? Are there other sources of $Ca^{2+}$ involved? How does PKCα activate RhoA? What are the GEFs involved in the activation of RhoA?

In general, increased intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) implies either the release of calcium from the endoplasmic reticulum or uptake mediated by channels in the membrane. IP3-R inhibition blocks the effect of Thy-1 in astrocytes, and neither RhoA activation nor FA formation is observed in cells treated with this inhibitor (Henriquez et al. 2011). Surprisingly, this cellular response depends also on Thy-1-induced release of ATP from astrocytes, which stimulates influx of extracellular $Ca^{2+}$ through P2X7 purinergic receptors. The increase in $[Ca^{2+}]_i$ is dependent on the interaction of Thy-1 with integrins, because the mutant of the wild-type molecule containing an

inactive integrin-binding domain, Thy-1(RLE), has no effect. In addition, the increase in $[Ca^{2+}]_i$ mediated by the P2X7 receptor is required for the formation of FA (Henriquez et al. 2011). Taken together, these data indicate that both $Ca^{2+}$ from the endoplasmic reticulum via activation of IP3-R channels and uptake of extracellular $Ca^{2+}$ are required for the formation of FA induced by Thy-1 in astrocytes. Both the decrease in the expression of P2X7 and inhibition of IP3-R channel block the formation of FA, indicating that both $Ca^{2+}$ currents are necessary for the formation of FA (Henriquez et al. 2011). In retinal ganglion cells, Thy-1 has recently been shown to interact with HCN4, a cation channel subunit (Partida et al. 2012).

It has been suggested that Thy-1 interaction with $\beta_3$ integrin may activate bidirectional signaling inducing structural changes in $\beta_3$-expressing astrocytes, thus modulating neurite outgrowth of Thy-1-expressing neurons (Avalos et al. 2002). Indeed, recently published studies reported that the $\alpha_v\beta_3$ integrin serves as a ligand for Thy-1 in neurons, which upon binding induces Thy-1 to aggregate in the plasma membrane and leads to neurite outgrowth inhibition and retraction of already formed processes (Herrera-Molina et al. 2012). Astrocytes expressing $\alpha_v\beta_3$, but not those in which the $\beta_3$ subunit has been reduced by specific siRNA, inhibit neurite growth of primary cortical neurons maintained for 4–7 days in culture. Recombinant $\alpha_v\beta_3$ expressed as a fusion protein with the Fc portion of IgG1 binds to Thy-1-containing regions of neurons, but remains unbound when these cells have had surface Thy-1 removed by PI-PLC treatment. In addition, $\alpha_v\beta_3$-Fc has no effect on neurons that do not express Thy-1. On the other hand, addition of $\alpha_v\beta_3$-Fc to neurons that have been in culture for more than 13 days, showing a more differentiated phenotype, causes retraction of neuronal processes generating bulb-clubbed endings in these cells (Herrera-Molina et al. 2012).

The mechanisms and consequences of interaction between Thy-1 and $\alpha_v\beta_3$ and modulation of signaling are still unknown and require further investigation, because downregulation of Thy-1 expression or inhibition of Thy-1 signaling on mature neurons may facilitate nerve regeneration.

According to data from antibody cross-linking studies, Thy-1-induced neurite outgrowth requires calcium influx, activation of L- and N-type calcium channels, and G-protein signaling (Doherty et al. 1993). Thy-1 interacts with Fyn and Gαi family members in avian neurons and with α- and β-tubulin within lipid rafts (Henke et al. 1997). Thy-1 blocking antibody decreases kinase activity within isolated lipid rafts, and these signaling changes may contribute to Thy-1's effects on neurite outgrowth. In agreement with these data, Thy-1 engaged by its endogenous ligand, $\alpha_v\beta_3$, triggers intracellular signaling that involves the recruitment and inactivation of the non-receptor tyrosine kinase Src (Herrera-Molina et al. 2012); see also Sect. 1.6.

## 1.8 Thy-1 Adhesive Signaling

Thy-1 can modulate cell signaling "in *trans*" (heterotypically) by Thy-1 on one cell engaging a Thy-1 ligand on another cell. This phenomenon is illustrated by human dermal microvascular endothelial cell (HDMEC) Thy-1 binding to $\alpha_X\beta_2$ (p150, 95,

CD11c/CD18) or $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) on leukocytes, promoting their adhesion and transendothelial migration (Choi et al. 2005; Wetzel et al. 2004; Saalbach et al. 2000), as well as by melanoma cell $\alpha_v\beta_3$ binding to Thy-1 on activated endothelium (Saalbach et al. 2002, 2005), which may promote melanoma metastasis, and by astrocyte–neuron interactions as described above. A number of novel findings are reported in the latter case. The β chain of the $\alpha_v\beta_3$ immunoprecipitated from DI TNC1 rat astrocytes is of smaller-than-expected molecular size, suggesting alternative splicing, posttranslational modification, or increased sensitivity to proteolytic enzymes. However, full-length $\alpha_v\beta_3$ is known to bind Thy-1 in vitro. The RLD sequence in Thy-1 is required for binding to $\alpha_v\beta_3$ integrin and occurs within a highly conserved region (Hermosilla et al. 2008), similar to RGD, the $\alpha_M\beta_2$-binding region in fibrinogen (Altieri et al. 1993). Thy-1 signaling via $\alpha_v\beta_3$ involves FAK and RhoA GTPase, the same pathways activated by "in *cis*" (homotypic) Thy-1 signaling in pulmonary fibroblasts (Barker et al. 2004a, b; Rege et al. 2006). Thy-1 *cis* signaling also occurs in neurons [neurite outgrowth inhibition; Herrera-Molina et al. (2012)]; however, the Thy-1-interacting molecules within neurons, and the downstream signaling pathways activated, have yet to be identified. Others have shown that neurite outgrowth inhibition requires the Thy-1-specific GPI anchor and lipid raft integrity (Tiveron et al. 1992).

Integrity of lipid raft micro- and nano-domains appears important to Thy-1 *cis* signaling. Rafts are microdomains enriched in cholesterol, phosphatidylcholine, and sphingolipids that have been associated with the actin cytoskeleton constituting a platform for signal transduction and communication of the extracellular and the cell interior (Chen et al. 2009a). Thy-1 has been shown to have equal mobility in lipid rafts as it does in the rest of the plasma membrane, which facilitates its trafficking into and out of lipid rafts, whereas transmembrane proteins are less mobile and more spatially constrained (Zhang et al. 1991). Replacement of the GPI anchor with a membrane-spanning domain or disruption of lipid rafts by cholesterol depletion abrogates Thy-1-mediated *cis* signaling (Rege et al. 2006; Tiveron et al. 1992). The residence time of Thy-1 within rafts is controlled by interaction with the cytoskeleton (Chen et al. 2006). To convey messages to the cell interior, Thy-1 clusters in the plasma membrane interacting with itself, adaptors, transducers, or signaling molecules, placing Thy-1 as an important part of a complex that triggers signaling events to the cell interior. In the early 1990s, the presence of multimeric forms of Thy-1 predominantly in differentiated neuron-like cell lines and primary neurons was described. It has been suggested also that these multimers might stabilize the complex formed between Thy-1 and the cytoskeleton (Mahanthappa and Patterson 1992). Thy-1 thus appears to regulate the trafficking and partitioning of signaling molecules into and out of lipid raft domains, thereby modulating signaling networks associated with the cytoskeleton. In this way Thy-1 may act alternately as an inhibitor of signaling molecules such as SFKs, by sequestering them, or as a facilitator of signaling, by regulating their trafficking.

Thy-1 also associates with proteins in the inner leaflet of the plasma membrane via myristoylated and palmitoylated posttranslational modifications, such as the

scaffold proteins reggie1 and reggie2 (Neumann-Giesen et al. 2004) and SFKs, as discussed above (Bradshaw 2010). Reggies are key modulators of neuronal process extension and the cytoskeleton, whereas SFKs are non-receptor tyrosine kinases responsible for initiation of cell signaling via tyrosine phosphorylation in response to signals internalized via Thy-1 (Chen et al. 2005, 2006, 2009b; Deininger et al. 2003; Herrera-Molina et al. 2012). Therefore, Thy-1 clusters, lipids, and signaling proteins such as reggies and SFKs are part of these signaling cascades generating signal transduction pathways in *cis*. Other important players in these signaling complexes are Thy-1 transmembrane transducers, which, as indicated earlier, can establish a communication between proteins present in the outer leaflet of the cell membrane with those located in the inner leaflet of the bilayer.

Additional studies are required to better characterize Thy-1 interaction with other molecules in regulation of signaling, particularly in *cis*. Its role in regulating Rho GTPases, focal adhesion turnover, and stress fiber formation is suggestive that Thy-1 interacts in *cis* with integrins, but this has not been shown definitively. Furthermore, the mechanisms regulating Thy-1 localization and trafficking to specialized and distinct lipid nano-domains have not been defined.

## 1.9 Conclusions

Thy-1 is expressed by a diversity of cell types and has variable effects on cell phenotypes. Hematopoietic and stromal stem cells in an undifferentiated state express Thy-1, whereas in neurons, Thy-1 is developmentally regulated and associated with cessation of neurite outgrowth. In the nervous system, Thy-1 is known to modify the phenotypes of neurons and astrocytes and to mediate their interaction, functioning as an adhesion molecule via interactions with integrins and Syndecan-4. It is known that Thy-1 regulates signaling both in *trans* and in *cis*. Thy-1 is known to interact also with itself, the reggie proteins, and SFK and to modulate intracellular signaling despite lacking a transmembrane domain. Much remains unclear regarding the molecular mechanisms by which Thy-1 modulates intracellular signaling. It is clear, however, that both the expression of Thy-1 and its effects depend a great deal on the cellular and tissue context. Increased understanding of mechanisms of Thy-1 signaling and regulated expression could allow therapeutic manipulation of cell phenotypes in nerve injury, malignancy, and fibrotic disorders.

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