TAM Receptor Tyrosine Kinases: Biologic Functions, Signaling, and Potential Therapeutic Targeting in Human Cancer

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Tyro-3, Axl, and Mer constitute the TAM family of receptor tyrosine kinases (RTKs) characterized by a conserved sequence within the kinase domain and adhesion molecule-like extracellular domains. This small family of RTKs regulates an intriguing mix of processes, including cell proliferation/survival, cell adhesion and migration, blood clot stabilization, and regulation of inflammatory cytokine release. Genetic or experimental alteration of TAM receptor function can contribute to a number of disease states, including coagulopathy, autoimmune disease, retinitis pigmentosa, and cancer. In this chapter, we first provide a comprehensive review of the structure, regulation, biologic functions, and downstream signaling pathways of these receptors. In addition, we discuss recent evidence which

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suggests a role for TAM receptors in oncogenic mechanisms as family members are overexpressed in a spectrum of human cancers and have prognostic significance in some. Possible strategies for targeted inhibition of the TAM family in the treatment of human cancer are described. Further research will be necessary to evaluate the full clinical implications of TAM family expression and activation in cancer.

I. INTRODUCTION

Receptor tyrosine kinases (RTKs) are transmembrane proteins which transduce signals from the extracellular environment to the cytoplasm and nucleus. In this manner, RTKs regulate normal cellular processes, including survival, growth, differentiation, adhesion, and motility. Abnormal expression or activity of RTKs can render them transforming in cellular and animal models. Furthermore, increased RTK expression or activation has been directly implicated in the pathogenesis of myriad human cancers leading to intense interest in the development and testing of tyrosine kinase inhibitors as cancer therapeutics.

The 58 RTKs in the human genome are classified into 20 families by amino acid sequence identity within the kinase domain and structural similarities within their extracellular regions (Robinson *et al.*, 2000). The focus of this review is the TAM family which includes *T*yro-3, *A*xl, and *M*er, three receptors which share the vitamin K-dependent ligands Gas6 and Protein S. Signaling pathways employed by the TAM family have been recently elucidated and shown to mediate diverse cellular functions, including macrophage clearance of apoptotic cells, platelet aggregation, and natural killer (NK) cell differentiation. This review will highlight the role of these RTKs in normal cellular function as well as the mechanisms employed by the TAM family to promote oncogenesis. In addition, we will discuss possible means of targeted inhibition of the TAM family in the treatment of human cancer.

II. MOLECULAR BIOLOGY OF TAM RECEPTORS

Like all RTKs, Tyro-3, Axl, and Mer contain an extracellular domain, a transmembrane domain, and a conserved intracellular kinase domain. The TAM family is distinguished from other RTKs by a conserved sequence, KW(I/L)A(I/L)ES, within the kinase domain and adhesion molecule-like domains in the extracellular region (Fig. 1A). More specifically, two immunoglobulin-like (Ig) domains and two fibronectin type III (FNIII) domains comprise nearly the entire ectodomain of each family member. These motifs are thought to be important in cell–cell contacts and mimic the structure of

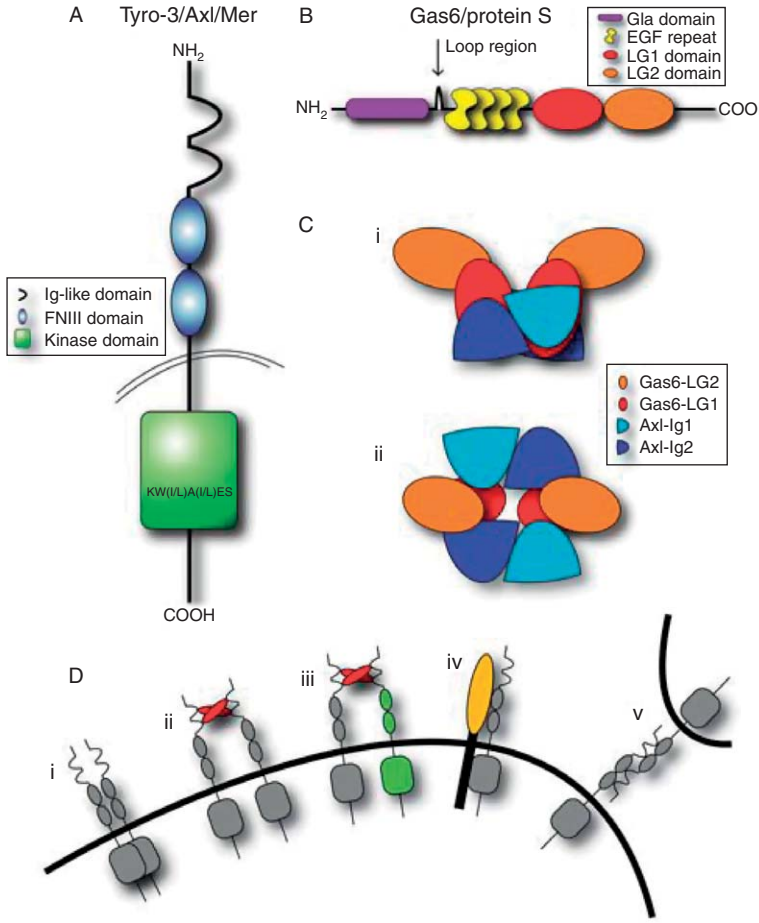

Fig. 1 Structure, binding, and activation of TAM receptors are their ligands. (A) Domain organization of Tyro-3, Axl, and Mer. The conserved sequence within the kinase domain is indicated. (B) Domain structure of the TAM receptor ligands, Gas6 and Protein S. Protein S contains thrombin cleavage sites in the loop region and has not been shown to activate Axl. (C) Axl binds to Gas6 with 2:2 stoichiometry as shown from the side (i) and from the top (ii). No ligand/ligand or receptor/receptor contacts were observed in crystals of the minimal complex containing the two LG domains of Gas6 and the two Ig domains of Axl. (D) Possible means of TAM receptor activation include: (i) ligand-independent dimerization, (ii) ligand-dependent dimerization, (iii) heteromeric dimerization of two different TAM receptors, (iv) heterotypic dimerization with a non-TAM receptor, and (v) trans-cellular binding of extracellular domains.

neural cell adhesion molecule (NCAM), which contains five Ig domains and two FNIII domains (Yamagata *et al.*, 2003). Among the RTKs, Tie (Tie1) and Tek (Tie2) are the only other receptors that contain both Ig and FNIII extracellular domains. The FGF, VEGF, and PDGF growth factor receptor families contain Ig domains while the Ephrin and Insulin families contain FNIII domains. Although the TAM receptors share extracellular motifs with the above RTKs, the MET RTK family (composed of Met and Ron) is most closely related to the TAM family on the basis of amino acid sequence of the kinase domain (Robinson *et al.*, 2000). The MET and TAM receptors activate common signaling molecules resulting in similar functions of the two RTK families (Birchmeier *et al.*, 2003; Hafizi and Dahlback, 2006a). Thus, both the extracellular domain and the intracellular kinase domain are important determinants of the cellular processes regulated by specific RTKs.

The TAM receptor genes share similar genomic structure encoding transcripts which range in size from 3 to 5 kb (Graham *et al.*, 1994, 1995; Mark *et al.*, 1994; O'Bryan *et al.*, 1991). Within the TAM family, Tyro-3 and Axl appear to have the most similar genomic structure sharing the same number, 20, and size of exons (Lewis *et al.*, 1996b; Lu *et al.*, 1999; Schulz *et al.*, 1993). Mer is encoded by 19 exons (Gal *et al.*, 2000). Axl and Tyro-3 contain alternative splice sites, although the location and outcome of splicing are different. A splice variant of Mer has been suggested but not fully characterized (Graham *et al.*, 1995). Alternative splicing of Tyro-3 near the 5′ end results in three different splice variants containing either exon 2A, exon 2B, or exon 2C (Biesecker *et al.*, 1995; Lewis *et al.*, 1996b; Lu *et al.*, 1999). These exons encode a signal peptide, suggesting that the presence of these splice variants may impact posttranslational processing, localization, and/or function of Tyro-3. Two Axl variants have been observed resulting from alternative splicing of exon 10 (Neubauer *et al.*, 1994; O'Bryan *et al.*, 1991; Schulz *et al.*, 1993). This exon encodes part of the second FNIII domain just upstream from the transmembrane region (Lu *et al.*, 1999). It remains unknown whether the Tyro-3 and Axl variants are produced from a single transcript or from multiple promoters. However, analysis of Axl and Mer sequences upstream of their respective translation initiation sites revealed a GC-rich promoter region lacking traditional TATA or CAAT boxes (Schulz *et al.*, 1993; Wong and Lee, 2002). Further analysis of the Mer promoter suggests that several transcription factors, including Sp1, Sp2, and E2F, may regulate promoter activity (Wong and Lee, 2002).

In contrast to the striking similarity of genomic structure between Tyro-3 and Axl, Axl and Mer have the most similar tyrosine kinase domain amino acid sequence (Graham *et al.*, 1995; Robinson *et al.*, 2000). Overall, the protein sequences of the human TAM receptors share 31–36% identical (52–57% similar) amino acids within the extracellular region. The intracellular

domains share 54–59% sequence identity (72–75% similarity) with higher homology in the tyrosine kinase domain (Graham *et al.*, 1995). The full-length Tyro-3, Axl, and Mer proteins contain 890, 894, and 999 amino acids, respectively. Although the predicted protein sizes are 97, 98, and 110 kDa for Tyro-3, Axl, and Mer, respectively, the actual molecular weights range from 100 to 140 kDa for Axl and Tyro-3 and 165–205 kDa for Mer due to posttranslational modifications, including glycosylation, phosphorylation, and ubiquitination (Lu *et al.*, 1999; O'Bryan *et al.*, 1991; Sather *et al.*, 2007; Valverde, 2005). Such modifications are possible mediators of tissue- and cell type-specific variations in TAM receptor function (Heiring *et al.*, 2004; Ling *et al.*, 1996) (see Section II.D).

A. Cloning/Nomenclature

In addition to sequence and structural similarities, the TAM receptor kinases are unusual in that the entire family was discovered within a span of 3 years. In the early 1990s, each TAM receptor gene was cloned from multiple species by independent groups resulting in confusing nomenclature (Table I). Axl was first detected in 1988 as an unidentified transforming gene in two patients with chronic myelogenous leukemia (CML) (Liu *et al.*, 1988). Three years later, two independent groups reported cloning of the human gene from patients with CML (O'Bryan *et al.*, 1991) and chronic myeloproliferative disorder (Janssen *et al.*, 1991). One group named the gene Axl from the Greek word for uncontrolled, anexelekto (O'Bryan *et al.*, 1991), and the other called the gene UFO indicating the unknown function of its protein product (Janssen *et al.*, 1991). Around the same time, a third group cloned the murine gene and named it Ark (adhesion-related kinase)

Table I TAM Receptor Nomenclature

Kinase	Synonyms	References
Tyro-3	Brt (m), Dtk (m), Rse, Sky, Tif, Etk-2 (m), Rek (ch)	Biesecker *et al.* (1993), Biscardi *et al.* (1996), Crosier *et al.* (1994), Dai *et al.* (1994), Fujimoto and Yamamoto (1994), Lai and Lemke (1991), Lai *et al.* (1994), Mark *et al.* (1994), Ohashi *et al.* (1994), Polvi *et al.* (1993)
Axl	Ark (m), Ufo, Tyro-7 (r)	Janssen *et al.* (1991), Lai and Lemke (1991), Liu *et al.* (1988), O'Bryan *et al.* (1991), Rescigno *et al.* (1991)
Mer	Eyk (ch), MerTK, Nyk, Tyro-12 (r)	Graham *et al.* (1994), Graham *et al.* (1995), Jia *et al.* (1992), Jia and Hanafusa (1994), Lai and Lemke (1991), Ling and Kung (1995)

ch, chicken; m, mouse; r, rat.

(Rescigno *et al.*, 1991). In the same year, 13 novel PCR fragments comprising 50–60 amino acids of the conserved tyrosine kinase catalytic domain were isolated from rat brain and named Tyro-1 to -13 (Lai and Lemke, 1991). Interestingly, the authors grouped Tyro-3, Tyro-7, and Tyro-12 into a novel subfamily based on the unique amino acid sequence found in their kinase domains. It would later be discovered that Tyro-7 is the same gene as Axl/UFO, Tyro-12 is the same gene as Mer, and Tyro-3 constituted the third member of the TAM family.

In 1992, a second member of the TAM family, v-ryk, was isolated from the chicken retrovirus RLP30 (Jia *et al.*, 1992). The cellular protooncogene, c-ryk, was later cloned from embryonic chicken brain and renamed c-eyk in order to avoid confusion with an unrelated tyrosine kinase also called ryk (Jia and Hanafusa, 1994). Later that same year, our lab cloned the human gene from a B-lymphoblastoid λgt11 expression library and named it c-*mer* because it was found in *m*onocytes as well as in *e*pithelial and *r*eproductive tissues (Graham *et al.*, 1994). We cloned murine c-mer the following year (Graham *et al.*, 1995). The human gene was cloned by a separate group and called Nyk for NCAM-related tyrosine kinase (Ling and Kung, 1995). Mer was also named MerTK for Mer tyrosine kinase in a paper which mapped the human gene to chromosome 2q14.1 (Weier *et al.*, 1999).

In addition to the earlier mentioned PCR fragment isolated from rat (Lai and Lemke, 1991), fragments of murine Tyro-3, called Etk-2 (Biesecker *et al.*, 1993), and human Tyro-3 (Polvi *et al.*, 1993) were cloned from mouse embryonic stem cells and human teratocarcinoma cell, bone marrow, and melanocyte cDNA libraries, respectively. In 1994, the murine and human genes were cloned by multiple labs. The murine gene was named Dtk (Crosier *et al.*, 1994), Brt (Fujimoto and Yamamoto, 1994), Rse (Mark *et al.*, 1994), and Tyro-3 (Lai *et al.*, 1994) while the human gene was called Sky (Ohashi *et al.*, 1994), Tif (Dai *et al.*, 1994), or Rse (Mark *et al.*, 1994). Subsequent sequence analysis revealed that Dtk and Brt were alternative splice variants (Lewis *et al.*, 1996b). The chicken ortholog was cloned in 1996 but was given the name Rek because of limited amino acid sequence identity with the mouse and human genes (66% and 68%, respectively) (Biscardi *et al.*, 1996).

While many of these names were used initially in the literature, Tyro-3, Axl, and Mer (or MerTK) have become the most commonly published and will be used exclusively throughout the remainder of this review.

B. Expression Patterns

Although expression of TAM receptor mRNA has been observed in embryonic tissues (Crosier *et al.*, 1996; Faust *et al.*, 1992; Graham *et al.*, 1995; Lai and Lemke, 1991), single, double, and even triple knockouts are

viable without obvious signs of developmental defect at birth (Lemke and Lu, 2003; Lu and Lemke, 2001; Lu *et al*., 1999). These data suggest that the TAM RTKs are largely nonessential for embryogenesis. Conversely, TAM adult knockout mice develop diverse phenotypes in a wide range of tissues revealing some of the most prominent cellular functions of TAM receptors (discussed in Section II.E).

In adult tissues, Tyro-3, Axl, and Mer exhibit widespread distribution with overlapping but unique expression profiles. Tyro-3 is most abundantly expressed in the nervous system, and is also found in ovary, testis, breast, lung, kidney, osteoclasts, and retina as well as a number of hematopoietic cell lines including monocytes/macrophages and platelets (Angelillo-Scherrer *et al*., 2001; Katagiri *et al*., 2001; Lai *et al*., 1994; Lu and Lemke, 2001; Mark *et al*., 1994; Prasad *et al*., 2006). Axl is expressed ubiquitously (O'Bryan *et al*., 1991), with notable levels found in the hippocampus and cerebellum (Bellosta *et al*., 1995) as well as monocytes/macrophages, platelets, endothelial cells, heart, skeletal muscle, liver, kidney, and testis (Angelillo-Scherrer *et al*., 2001; Graham *et al*., 1995; Neubauer *et al*., 1994). Within the hematopoietic lineages, Mer is expressed in monocytes/macrophages, dendritic cells, NK cells, NKT cells, megakaryocytes, and platelets (Angelillo-Scherrer *et al*., 2001; Behrens *et al*., 2003; Graham *et al*., 1994). High levels of Mer expression are also detected in ovary, prostate, testis, lung, retina, and kidney. Lower levels of Mer are found in heart, brain, and skeletal muscle (Graham *et al*., 1994, 1995; Prasad *et al*., 2006). Tyro-3, Axl, and Mer also display ectopic or overexpression in numerous cancers, including myeloid and lymphoblastic leukemias, melanoma, breast, lung, colon, liver, gastric, kidney, ovarian, uterine, and brain cancers (Table II). However, the pattern differs for each family member, e.g. Mer is found in lymphoid leukemia while Axl is not (Graham *et al*., 1994, 2006; Neubauer *et al*., 1994).

C. Ligands and Crystal Structures

The vitamin K-dependent protein Gas6 was first identified as a ligand for Axl in 1995 (Stitt *et al*., 1995; Varnum *et al*., 1995). The related vitamin K-dependent anticoagulation factor, Protein S, was described as a ligand for Tyro-3 (Stitt *et al*., 1995). Although numerous subsequent studies confirmed that Gas6 binds to and activates all three members of the TAM receptor family, the validity of Protein S as a ligand for any of the TAM receptors became subject to extensive debate (Chen *et al*., 1997; Godowski *et al*., 1995; Mark *et al*., 1996; Nagata *et al*., 1996; Ohashi *et al*., 1995). At the heart of the dispute was the issue of physiological relevance as the initial study used human Protein S to activate murine Tyro-3. Further studies were

Table II TAM Receptor Expression in Human Cancers

Cancer	Axl	Mer	Tyro-3	References
Myeloid leukemias (AML, CML)	+		+	Challier *et al.* (1996), Crosier *et al.* (1995), Liu *et al.* (1988), Neubauer *et al.* (1994), Rochlitz *et al.* (1999)[a]
Lymphoid leukemias (ALL)		Ect		Graham *et al.* (1994), Graham *et al.* (2006), Yeoh *et al.*, (2002)
Erythroid leukemia	+			Challier *et al.* (1996)
Megakaryocytic leukemia	+			Challier *et al.* (1996)
Mantle cell lymphoma		+		Ek *et al.* (2002)
Multiple Myeloma			+	De Vos *et al.* (2001)
Uterine endometrial cancer	+			Sun *et al.* (2003)
Gastric cancer	+	+		Lin *et al.* (1999), Wu *et al.* (2002)[b]
Colon cancer	+			Craven *et al.* (1995)
Prostate cancer	+	+		Jacob *et al.* (1999), Mahajan *et al.* (2005), Sainaghi *et al.* (2005), Wu *et al.* (2004)
Thyroid cancer	+			Ito *et al.* (1999, 2002), Tanaka *et al.* (1998)
Lung cancer	+			Shieh *et al.* (2005),[c] Wimmel *et al.* (2001)
Breast cancer	+	+		Berclaz *et al.* (2001), Meric *et al.* (2002), Zantek *et al.* (2001), Tavazoie *et al.*, (2008)
Ovarian cancer	+			Macleod *et al.* (2005), Sun *et al.* (2004)
Liver cancer	+			Tsou *et al.* (1998)
Renal cell carcinoma	+			Chung *et al.* (2003)
Astrocytoma/Glioblastoma	+			Vajkoczy *et al.* (2006)
Pituitary adenoma		+		Evans *et al.* (2001)
Melanoma	+	+		Gyorffy and Lage (2007), Quong *et al.* (1994), van Ginkel *et al.* (2004)
Osteosarcoma	+			Nakano *et al.* (2003)
Rhabdomyosarcoma		+		Khan *et al.* (1999)

[a]Overexpression of Axl correlated with poor prognosis.
[b]Coexpression of Axl and Mer correlated inversely with patient prognosis.
[c]Overexpression of Axl correlated with metastatic cancer and poor prognosis.
Over- (+) or ectopic expression (Ect) of TAM receptors has been reported in numerous human cancers.

unable to demonstrate that Protein S could activate a TAM receptor of the same species, possibly due to the need for additional cofactor(s) or modification of the Protein S ligand. However, it was recently determined that purified recombinant murine Protein S does bind to and activate both endogenous murine Mer and heterologously expressed murine Tyro-3 (Prasad *et al.*, 2006). There is currently no evidence that Protein S activates

Axl. A large number of additional studies have investigated the interspecies affinities of Gas6 and Protein S for TAM receptors (reviewed in Hafizi and Dahlback, 2006b). Studies which evaluated the K_d values for human Gas6 binding to each of the three human TAM receptors *in vitro* suggest that Axl and Tyro-3 bind Gas6 with roughly equal affinity while Mer affinity for Gas6 is 3–10-fold lower (Chen *et al*., 1997; Fisher *et al*., 2005).

Gas6 and Protein S share 43% amino acid sequence identity and have the same domain structure with the exception of thrombin cleavage sites which are present in Protein S but not Gas6 (Dahlback and Villoutreix, 2005; Stenflo *et al*., 1987) (Fig. 1B). The N-terminal domain contains glutamic acid residues which must be carboxylated in a vitamin K-dependent reaction before Gas6 and Protein S are biologically active (Stenhoff *et al*., 2004). The γ-carboxyglutamic acid (Gla) domain is followed by four EGF-like repeats and two C-terminal globular laminin G-like (LG) domains. The Gla domain mediates Ca^{2+}-dependent binding to negatively charged membrane phospholipids exposed on the surface of apoptotic cells. The LG domains form a V-shaped structure stabilized by a calcium-binding site and mediate ligand–receptor interactions (Mark *et al*., 1996; Sasaki *et al*., 2002). Solution for the crystal structure of a Gas6 fragment containing the two LG domains revealed an unusual α-helix within LG2 located at the edge of the β-sandwich fold typical of all LG domains. In addition, five amino acids within LG2 constitute a patch of surface-exposed hydrophobic residues located near the crook of the "V" created by LG1 and LG2. These residues are also in close proximity to the stabilizing calcium-binding site. It has not been determined whether the calcium-binding site contributes to RTK binding. Mutagenesis studies and receptor activation assays suggested that the hydrophobic residues within LG2 comprise at least part of the Axl binding site (Sasaki *et al*., 2002). However, LG2 alone does not bind to or activate Axl, and a later study by the same group determined that only LG1 of Gas6 binds Axl (Sasaki *et al*., 2006). The authors suggest that the hydrophobic residues may still affect ligand/receptor binding indirectly. Direct binding between Axl and the LG1 domain of Gas6 was first demonstrated by Fisher *et al*. (2005). An anti-Gas6 monoclonal antibody diminished Gas6 binding to Axl and the antibody binding epitope was mapped to residues 403–414 within the J–K loop of LG1. Notably, this region is located near the edge of the LG1 β-sandwich fold, distant from the hydrophobic patch within LG2.

The crystal structure of a Gas6/Axl complex finally revealed that the LG1 domain of Gas6 makes two separate contacts with the IG1 and IG2 domains of Axl (Sasaki *et al*., 2006). Each contact is characterized by antiparallel alignment of edge β-strands such that continuous β-sheets span the molecular junction. Interestingly, no ligand/ligand or receptor/receptor contacts were reported in this minimal complex containing the LG domains of Gas6 and the Ig domains of Axl (Fig. 1C). Additional experiments suggest

that ligand-mediated TAM receptor dimerization occurs via a two-step mechanism whereby one molecule of Gas6 binds one receptor molecule with high affinity at the LG1/IG1 "major" contact. Lateral diffusion of these 1:1 ligand/receptor complexes results in dimerization of two 1:1 complexes via the LG1/IG2 "minor" contact. Thus, a 2:2 ligand/receptor complex is formed. Further evidence to support two Gas6/Axl binding sites was provided by receptor binding studies, which demonstrated that Gas6 can simultaneously bind Axl–Fc and a neutralizing Gas6 antibody (Fisher *et al.*, 2005). Receptor binding studies of an N-terminal fragment of Tyro-3 demonstrated that one site of Gas6/Tyro-3 receptor interaction is localized to the two Ig domains. Although the crystal structure of the Tyro-3 fragment and sequence alignment of the three TAM receptors predict the existence of a Gas6-binding site near the interface of the two Ig domains, no empirical evidence regarding the actual ligand binding site(s) was provided (Heiring *et al.*, 2004). Thus, additional studies are required to determine whether Tyro-3 and Mer bind Gas6 in the same manner as does Axl. Given that there is no current information describing Protein S as a ligand for Axl, it will be particularly interesting to see how Protein S interacts with Mer and Tyro-3.

Until recently, no structural information was available for the kinase domains of TAM receptors. The crystal structure of the catalytic domain of human Mer has been solved and may provide new insight into numerous aspects of TAM receptor biology, including mechanisms of receptor activation and interaction with downstream signaling molecules (Walker *et al.*, 2007).

D. Regulation of Receptor Kinase Activity

1. CONVENTIONAL ACTIVATION

Typical activation of RTKs involves ligand binding to the extracellular domain (Schlessinger, 2000). Ligand binding induces receptor dimerization and subsequent trans-autophosphorylation of tyrosine residues within the cytoplasmic domain (Fig. 1D). The result of autophosphorylation is two-fold: (1) increased catalytic efficiency leads to phosphorylation of other substrates and (2) tyrosine-phosphorylated RTKs and other proteins constitute docking sites that recruit signaling molecules containing SH2, PTB, or other phosphotyrosine-binding domains. This allows RTKs and other proteins to form macromolecular signaling complexes. For Mer, three tyrosine residues (Y-749, Y-753, and Y-754 in the human sequence) within the activation loop of the kinase domain have been identified as the primary sites of autophosphorylation (Ling *et al.*, 1996). Interestingly, *in vitro* kinase

assays utilizing peptides with two of the three tyrosines mutated to phenylalanine residues as substrates for WT Mer demonstrated that tyrosine 749 is the preferred site of autophosphorylation. Additional *in vitro* kinase assays evaluated WT Mer versus mutant Mer phosphorylation of a synthetic peptide containing tyrosines 749, 753, and 754. Single mutations of tyrosines 749, 753, and 754 to phenylalanine reduced Mer kinase activity to 39%, 10%, and <6% of WT Mer, respectively, suggesting that all three residues are required for complete functional activity of the kinase (Ling *et al*., 1996). These three tyrosines are conserved among the TAM receptors and correspond to residues 681, 685, and 686 in the human sequence on Tyro-3 and residues 698, 702, and 703 in the human sequence of Axl. Autophosphorylation of Tyro-3 and Axl have not been reported at these residues.

Three alternative tyrosine residues (Y-779, Y-821, and Y-866) within the C-terminal domain of Axl have been proposed as potential autophosphorylation sites (Braunger *et al*., 1997). These three sites, and in particular Y-821, mediate interaction of Axl with a number of signaling molecules including phospholipase C (PLC), phosphatidyl inositol 3 kinase (PI3K), and Grb2 (Braunger *et al*., 1997; Fridell *et al*., 1996). All of the interactions identified were dependent on Axl tyrosine kinase activity; however, the studies do not provide clear evidence that tyrosine residues 779, 821, and 866 are indeed sites of autophosphorylation. The residue equivalent to Axl Y821 in Mer (Y-867/872 in the murine/human sequences) is also a probable site of interaction with multiple signaling molecules. Mutation of tyrosine 867/872 to phenylalanine did not reduce tyrosine phosphorylation of Mer, suggesting that this site does not regulate kinase activity efficiency (Georgescu *et al*., 1999). Furthermore, Axl mutants lacking tyrosine 821 display normal ligand-induced tyrosine phosphorylation (Fridell *et al*., 1996). Alternative to these tyrosines being sites of autophosphorylation, they may be phosphorylated by another kinase recruited by autophosphorylation at different residues. Src-family non-RTKs (SFKs) are potential candidates for this activity as they have been shown to interact with both Axl and Tyro-3 (Braunger *et al*., 1997; Toshima *et al*., 1995). The combination of site-directed mutagenesis and *in vitro* kinase activity assays allows more definitive assignment of tyrosines 749, 753, and 754 as Mer autophosphorylation sites (Ling *et al*., 1996). However, it remains possible that these and additional tyrosine or serine/threonine residues are phosphorylated by other kinases. It is also possible that a unique complement of residues becomes phosphorylated in response to specific stimuli within the cellular microenvironment. Expression of TAM receptors in certain cell types may also lead to distinct phosphorylation patterns. Future generation of phosphosite-specific antibodies will greatly aid our ability to address these types of questions.

2. ATYPICAL ACTIVATION

In some cases, ligand-independent receptor dimerization and activation can occur (Fig. 1D). For example, overexpression of Axl leads to cell aggregation via homophilic binding of the extracellular domains on neighboring cells (Bellosta *et al.*, 1995). Although cell aggregation correlated with increased tyrosine phosphorylation of Axl, activation of the kinase domain was not required for homophilic binding (Bellosta *et al.*, 1995). Because the specific residue(s) responsible for the observed increase in tyrosine phosphorylation remain unknown, it is possible that phosphorylation occurred at a site unrelated to receptor activation. Studies of Axl and Tyro-3 overexpression suggest that these receptors are also capable of ligand-independent dimerization and autophosphorylation (Burchert *et al.*, 1998; Taylor *et al.*, 1995a). Further evidence to support ligand-independent dimerization was provided by crystal structures of a Tyro-3 fragment containing the two N-terminal Ig domains (Heiring *et al.*, 2004). Importantly, a distinction must be made between dimerization of two receptors on the surface of one cell and homophilic binding of receptors on neighboring cells (i versus v in Fig. 1D) as exogenous expression of Tyro-3 in Sf9 cells (Toshima *et al.*, 1995) and basal expression of Axl in NIH3T3 cells (Bellosta *et al.*, 1995) are not sufficient to induce homophilic binding. Thus, it remains unknown whether this phenomenon occurs with any endogenous TAM receptor.

An increasingly common theme in cell signaling literature is cross-talk between receptor systems. Ligand-independent heterotypic receptor dimerization of Axl with interleukin-15 receptor alpha (IL-15Rα) has been reported in immortalized and primary fibroblasts (Budagian *et al.*, 2005b) (Fig. 1D). Binding of IL-15 to IL-15Rα, not Axl, leads to Axl-mediated phosphorylation of IL-15Rα as well as Axl phosphorylation, although it is not known whether this is a direct action of the Axl kinase domain. Thus, IL-15 transactivates the Axl receptor and downstream signaling molecules, including PI3K, Akt, and ERK. Heterotypic dimerization of Axl with cytokine receptors seems to be specific to IL-15Rα as Axl does not coprecipitate IL-2, IL-4, IL-7, IL-9, or IL-21 receptor subunits, even in the presence of ligand (Budagian *et al.*, 2005b). To date, similar heterotypic receptor interactions have not been reported for Mer or Tyro-3.

Another unexplored possibility is an unusual heteromeric interaction among the three TAM receptors (Fig. 1D). Homo- and heterodimerization have been reported for other RTK families such as EGFR family members. Recent studies suggest that Gas6-mediated phosphorylation/activation of one TAM receptor may require the presence of one or both of the other TAM receptors in some circumstances (Angelillo-Scherrer *et al.*, 2005; Seitz *et al.*, 2007). Interestingly, Western blotting studies suggest that relatively equal amounts of Axl total protein can be detected in whole cell lysates of

platelets from WT and Tyro-3−/− mice. However, flow cytometry experiments demonstrated that surface expression of Axl is significantly reduced in Tyro-3−/− and Mer−/− mice (Angelillo-Scherrer *et al.*, 2005). Taken together, these data suggest that Axl may require the presence of Mer or Tyro-3 or both for functional surface delivery and stabilization within the plasma membrane.

3. MECHANISMS OF DEACTIVATION

Cellular control of RTK signal attenuation is important as aberrant or continued receptor signaling can lead to numerous pathological states, including cancer. Cells have developed numerous methods for inactivation of RTKs, including antagonistic ligands, hetero-oligomerization with kinase inactive mutants, phosphorylation of inhibitory residues by other kinases, dephosphorylation of activating residues by phosphatases, and receptor endocytosis accompanied by ligand dissociation, receptor degradation, or both (Schlessinger, 2000). Only a few of these pathways have been explored as possible mechanisms of TAM receptor regulation.

Many tyrosine kinases are negatively regulated by phosphorylation of an inhibitory residue. For example, phosphorylation of tyrosine 527 near the C-terminus of Src prevents activation of the kinase by promoting intramolecular binding to the SH2 domain, thus rendering the active site inaccessible. Interestingly, it has been postulated that tyrosine 866 on Axl, one of the same residues proposed as a site of autophosphorylation, might constitute an inhibitory phosphorylation site akin to C-terminal tyrosines found in SFKs and the EGFR (Burchert *et al.*, 1998). However, the same study concluded that the absence or mutation of this residue did not impact the ability of Axl-retroviruses to transform NIH3T3 cells. A second phosphorylation-mediated mechanism of receptor downregulation is receptor dephosphorylation by protein tyrosine phosphatases. The putative tyrosine phosphatase C1-TEN has been shown to bind Axl and overexpression of C1-TEN correlates with reduced cell survival, proliferation, and migration of 293 cells (Hafizi *et al.*, 2002, 2005b). Although neither enzymatic activity of C1-TEN nor direct dephosphorylation of Axl have been demonstrated, these results are consistent with C1-TEN-mediated Axl inactivation.

Soluble forms of Axl and Mer, produced by proteolytic cleavage and release of the ectodomain, can be detected in murine and human plasma (Budagian *et al.*, 2005a; Costa *et al.*, 1996; O'Bryan *et al.*, 1995; Sather *et al.*, 2007). Although a truncated form of Tyro-3 was found in the cytoplasm when expressed in 293 cells (Taylor *et al.*, 1995a), extracellular soluble Tyro-3 was not detected in human plasma (Sather *et al.*, 2007). Soluble Mer can also be produced by alternative splicing of the Mer transcript (our unpublished data). Although alternative splicing of Axl (O'Bryan *et al.*,

1991; Schulz *et al.*, 1993) and Tyro-3 (Biesecker *et al.*, 1995; Lewis *et al.*, 1996b) have been reported, the transcripts generated encode transmembrane proteins. Soluble TAM receptors bind to Gas6 and can act as a ligand sink and inhibit normal cellular functions of the full-length RTK (Budagian *et al.*, 2005a; Sather *et al.*, 2007). In the same regard, soluble TAM receptors may have therapeutic potential in pathological conditions, such as cancer, where TAM receptor activity is upregulated. This topic will be further explored in Section IV.

Evidence supporting endocytosis as a mechanism of TAM receptor downregulation was provided by a report which demonstrated that Gas6 stimulates interaction of Axl with the ubiquitin ligase c-Cbl and ubiquitination of Axl (Valverde, 2005), a process that has been demonstrated with other RTKs such as the EGFR. Clearly the study of mechanisms which regulate TAM receptor function and turnover is an area that needs further investigation.

E. Cellular Functions

Stimulation of TAM receptors can produce diverse cellular functions depending on the ligand–receptor combination as well as the cell type and microenvironment. Initial studies of individual TAM receptors suggested that each kinase performs unique functions in specific cell types. However, as the number of publications investigating two or three TAM receptors in the same system increases, it is becoming evident that the TAM receptors can serve overlapping and possibly cooperative roles. While it is beyond the scope of this review to discuss every cell type which expresses TAM receptors, several cellular functions of TAM receptors are discussed here according to specific cell types.

1. MACROPHAGES/DENDRITIC CELLS

TAM-receptor knockouts develop autoimmune diseases, including rheumatoid arthritis and lupus (Cohen *et al.*, 2002; Lemke and Lu, 2003). Loss of Mer alone confers susceptibility to autoimmunity (Scott *et al.*, 2001). However, the phenotype is more pronounced in double knockouts and most severe in triple knockouts (Lemke and Lu, 2003). These phenotypes likely result from accumulation of apoptotic cells and subsequent tissue necrosis combined with constitutive activation of the immune system. Studies of single, double, and triple mutants suggest that these defects are a result of TAM receptor loss from macrophages/dendritic cells (Lu and Lemke, 2001).

a. Clearance of Apoptotic Cells

Cell death via apoptosis is a necessary process for maintenance of normal cell number and health. Clearance of apoptotic cells plays an important role in many biological processes, including tissue development and homeostasis,

lymphocyte maturation, and pathological responses such as inflammation. Progressive accumulation of apoptotic cells leads to tissue necrosis and release of intracellular contents into the local environment. Because it is more difficult for immune cells to locate and clear this cellular debris, necrosis leads to inflammation and, in some cases, activation of autoantibody production.

Although a number of different types of professional phagocytes can ingest infectious microorganisms and particles, clearance of apoptotic cells is primarily mediated by macrophages and, to a lesser degree, dendritic cells. Because the surface of apoptotic cells and the phagocytes which digest them are both negatively charged, proteins must mediate the processes of cell recognition and engulfment. Specifically, apoptotic cells express phosphatidylserine (PS) on their surface, which has been shown to bind directly to phagocytes via PS receptors or indirectly via binding to one of several soluble proteins, including the TAM receptor ligands Gas6 and Protein S (Anderson *et al*., 2003; Nakano *et al*., 1997). Macrophages express all three TAM receptors (Graham *et al*., 1994; Lu and Lemke, 2001; Neubauer *et al*., 1994), suggesting a mechanism whereby TAM receptors and their ligands might mediate macrophage recognition of apoptotic cells.

Protein S binds to and stimulates phagocytosis of apoptotic cells (Anderson *et al*., 2003). However, there is currently no empirical evidence which directly correlates Protein S-mediated phagocytosis with activation of a TAM receptor. Conversely, *in vitro* studies demonstrated that Gas6 stimulates macrophage uptake of PS liposomes and uptake is blocked by the extracellular domain of Axl (Ishimoto *et al*., 2000). Similarly, soluble Mer bound to the Fc domain of human immunoglobulin G (Mer–Fc) inhibits macrophage phagocytosis of apoptotic cells presumably by sequestering Mer ligand (Sather *et al*., 2007). Several lines of evidence suggest that Mer is not required for binding to apoptotic cells but is essential for cell shape changes associated with engulfment of the apoptotic cell (Cohen *et al*., 2002; Guttridge *et al*., 2002; Hu *et al*., 2004; Scott *et al*., 2001; Todt *et al*., 2004). The TAM ligands are proposed to mediate phagocytosis of apoptotic cells by bridging an interaction between PS-expressing cells and TAM receptor-expressing macrophages. Thus, the tyrosine kinase domains of TAM receptors, in particular Mer, likely activate downstream signaling events, including integrins such as $\alpha v\beta 5$, which leads to cytoskeletal changes necessary for engulfment of apoptotic cells (Wu *et al*., 2005).

It is likely that unique mechanisms mediate clearance of apoptotic cells depending on the type of phagocyte involved and the tissue microenvironment. Accordingly, a recent study by Seitz *et al*. (2007) suggests that TAM receptor involvement in clearance of apoptotic cells varies according to cell and organ type. They found that Mer, and to a lesser degree Axl and Tyro-3, mediates macrophage clearance while dendritic cell clearance of apoptotic

cells is largely mediated by Axl and Tyro-3. These findings are consistent with an earlier study which showed that dendritic cells from mice lacking Mer protein exhibit normal phagocytosis of apoptotic cells (Behrens *et al.*, 2003).

One of the most intensely studied examples of TAM receptor-mediated macrophage clearance of apoptotic cells is phagocytosis of photoreceptor outer segment membranes by retinal pigment epithelium (RPE) cells. The role of Mer in RPE phagocytosis was initially elucidated through the study of the Royal College of Surgeons (RCS) rat, a widely studied model of recessively inherited retinal degeneration and animal model for the human disease retinitis pigmentosa. Two groups independently discovered that the genetic basis for RPE dysfunction in the RCS rat was due to a deletion of the second exon of Mer leading to aberrant transcription with a frameshift and translation termination signal 20 codons after the AUG (D'Cruz *et al.*, 2000; Nandrot *et al.*, 2000). In a similar manner, transgenic mice (Mer^{KD}) containing a truncated form of the Mer gene lacking the kinase domain exhibit total loss of Mer protein expression and a retinal phenotype similar to that of the RCS rat (Duncan *et al.*, 2003). Subsequent work demonstrated that loss of function mutations in human Mer are present in a small subset of patients with severe and progressive retinitis pigmentosa (Gal *et al.*, 2000; McHenry *et al.*, 2004; Thompson *et al.*, 2002). It would be interesting to determine whether these patients exhibit other similarities to Mer knockout mice, such as predisposition to autoimmune disease. Recent studies have demonstrated that viral gene transfer of Mer into the RCS rat retina results in correction of the RPE phagocytosis defect and preservation of photoreceptors, suggesting the exciting possibility of gene therapy for retinitis pigmentosa patients with Mer mutations (Tschernutter *et al.*, 2005; Vollrath *et al.*, 2001).

b. Cytokine Secretion

Cytokines are soluble proteins which mediate communication between cells of the immune system. Cytokines are released in response to extracellular stimuli, including microorganisms and antigens. A number of different cell types, including macrophages, secrete cytokines, and these soluble signaling molecules usually act over short distances. Cytokine levels indicate the status of the immune system and are subject to stringent regulation in order to avoid inappropriate immune responses. When cytokine levels are not held in check, constitutive activation of the immune system can occur resulting in development of autoimmunity. As mentioned previously, TAM receptor knockout mice develop autoimmune diseases likely due, at least in part, to abnormal regulation of cytokine release.

Mer^{KD} mice are more susceptible to lethal septic shock following lipopolysaccharide (LPS) challenge. LPS binds to surface receptors and activates nuclear factor (NF)-κB, which then initiates production of proinflammatory

cytokines, including TNFα. Pretreatment with anti-TNFα antibody protects against LPS-induced death, suggesting that TNFα is a key upstream regulator of lethal septic shock. Following LPS treatment, Mer^{KD} mice have elevated NFκB and TNFα levels relative to wild-type controls (Camenisch *et al.*, 1999). In addition, a recent study demonstrated that Mer activation stimulates the PI3K/Akt pathway which negatively regulates NFκB activation, thus decreasing TNFα production in dendritic cells (Sen *et al.*, 2007). These data suggest that one of the normal functions of Mer in macrophages and dendritic cells is attenuation of proinflammatory cytokine responses following exposure to bacterial endotoxin. TAM receptors may also mediate other antiinflammatory macrophage responses. For example, interferon (IFN) α has been shown to upregulate expression of Axl and Gas6 in human macrophages resulting in reduced TNFα production (Sharif *et al.*, 2006). A role for TAM receptors in a broad spectrum of antiinflammatory responses is further supported by the observation of hyperactive macrophages in TAM receptor triple knockouts which produce higher levels of the proinflammatory cytokine IL-12 than do wild-type counterparts (Lu and Lemke, 2001). TAM receptor regulation of the inflammatory response may be disrupted in various pathologies as microarray analysis of Mer kinase activation (via stimulation of FMS–Mer receptor chimera containing the extracellular domain of the M-CSF receptor and the transmembrane and cytoplasmic domains of Mer) in human prostate cancer cells indicated upregulation of proinflammatory cytokine genes, including IL-8, IL-11, and IL-24 (Wu *et al.*, 2004).

2. NATURAL KILLER CELLS

NK cells are lymphocytes which do not express any of the antigen receptors characteristic of T- or B-cells. NKT cells exhibit characteristics similar to both NK and T cells. Expression of Mer in both NK and NKT cells was first reported by Behrens *et al.* (2003), also demonstrating that the Mer tyrosine kinase domain is critical for normal cytokine release from NKT cells. A later study showed that NK cells also express Axl and Tyro-3 and all three TAM receptors are required for normal differentiation and functional maturation of NK cells (Caraux *et al.*, 2006).

3. PLATELETS

The first evidence to suggest a role for TAM receptors in platelet function came from studies of Gas6 knockout mice. Gas6−/− mice were protected against thrombosis and exhibited defective platelet aggregation (Angelillo-Scherrer *et al.*, 2001). In the same study, RT-PCR analysis demonstrated that platelets express Tyro-3, Axl, and Mer. A follow-up study used single

knockouts of Tyro-3, Axl, and Mer to demonstrate that all three receptors are required for normal platelet aggregation (Angelillo-Scherrer *et al.*, 2005). Loss of any one of the TAM receptors or application of soluble Axl protects against fatal thrombosis. These findings are supported by a study from our lab, which demonstrated that soluble Mer (Mer–Fc) reduces platelet aggregation *in vitro* and protects against collagen/epinephrine-induced thrombosis *in vivo* (Sather *et al.*, 2007). Furthermore, a recent study demonstrated that double and triple TAM receptor knockouts exhibit more severe impairment of platelet function than single knockouts (Wang *et al.*, 2007).

4. VASCULAR SMOOTH MUSCLE CELLS

Some of the first studies which evaluated cellular function of TAM receptors were conducted in vascular smooth muscle cells (VSMCs). In these early studies, expression of Axl and Gas6 was increased following vascular injury (Melaragno *et al.*, 1998). In additional experiments, Gas6 stimulation induced migration of Axl-overexpressing VSMCs (Fridell *et al.*, 1998). Furthermore, Gas6 protects VSMCs from apoptosis induced by serum starvation in an Axl kinase-dependent manner (Melaragno *et al.*, 2004). These results suggest that TAM receptors may play a role in vascular diseases, such as atherosclerosis, which are characterized by accumulation of VSMCs. Indeed, Gas6 has been shown to stimulate scavenger receptor expression in normal VSMCs (Murao *et al.*, 1999). Scavenger receptors facilitate uptake of low-density lipoprotein (LDL) which may lead to transformation of the VSMCs into foam cells and development of atherosclerosis. In advanced atherosclerotic lesions, however, TAM receptors may help slow the progression of disease by mediating ingestion of apoptotic macrophages and attenuating the proinflammatory response (Li *et al.*, 2006).

5. OTHER

Given their broad expression patterns, it is likely that TAM receptors perform important functions in numerous other cells types. For example, Tyro-3, Axl, Mer, and their mutual ligand Gas6 are all expressed in the central nervous system but their normal biological activity has not been widely studied in the brain (Lai and Lemke, 1991; Mark *et al.*, 1994; Prieto *et al.*, 1999, 2000). One exception is an established line of evidence demonstrating a role for Axl in survival and migration of gonadotropin-releasing hormone (GnRH) neurons (Allen *et al.*, 1999, 2002; Nielsen-Preiss *et al.*, 2007). Similarly, Gas6 has been shown to reduce cell death of Tyro-3-expressing hippocampal neurons following serum starvation (Funakoshi *et al.*, 2002). Taken together, these studies suggest that TAM receptors may

activate neurotrophic signaling pathways in specific regions of the central nervous system.

It also appears that the three TAM receptors act in concert to regulate spermatogenesis, as triple knockouts are infertile because of progressive degeneration of germ cells beginning one week prior to sexual maturity (Lu *et al*., 1999). The mechanism of germ cell death remains unknown except that it likely involves reduced communication between the TAM receptor-expressing Sertoli cells which line the seminiferous tubules and the interstitial Leydig cells which express Gas6 and Protein S. TAM receptor regulation of GnRH neurons may also contribute to the infertility of these knockouts as impaired migration of GnRH neurons inhibits sexual maturation.

F. TAM Receptor Signaling Pathways

The first hint towards understanding TAM receptor signaling came from studies of FMS–Mer receptor chimera by Ling and Kung in 1995. Around the same time, studies of EGF–Axl receptor chimera were published by an independent group (Fridell *et al*., 1996). When the studies began, the ligand for TAM receptors was unknown, necessitating the use of receptor chimera composed of, in the latter report, the EGFR receptor ectodomain and transmembrane domain fused to the intracellular kinase domain of Axl. During the course of the studies, Gas6 was discovered as a ligand for Axl and Tyro-3 and additional work was conducted with the native Axl receptor. Two important findings came out of this seminal work. First, signaling pathway(s) downstream from the Mer and Axl kinase domains were determined to include PI3K, Ras, and ERK. Second, studies of the Axl receptor chimera compared to the native Axl RTK demonstrated that variation in the extracellular domain has a significant impact on downstream signaling.

In the 12 years since, an abundance of research has been conducted with the goal of outlining signaling pathways downstream of TAM receptors. Most of these experiments utilize Gas6 to stimulate TAM receptor function but discuss relevance to only one TAM receptor, usually Axl. It should be noted that Gas6 will also activate other TAM receptors endogenously expressed by the cells under investigation. For example, all three TAM receptors are expressed in platelets and are required for normal function of these cells (see Section II.E.3). The downstream signaling pathway whereby TAM receptors mediate platelet aggregation likely involves cross-talk with the integrin family of receptors as platelets from TAM receptor knockouts exhibit impaired spreading after adhesion to fibrinogen. Indeed, Gas6 stimulates phosphorylation of β_3 integrin, PI3K, and Akt in resting platelets from WT, but not TAM receptor knockout mice (Angelillo-Scherrer *et al*.,

2005). Importantly, the specific contributions of each TAM receptor to this signaling pathway have yet to be clarified.

To avoid uncertainty regarding which TAM receptor is responsible for the observed effects, some studies have continued to use the receptor chimera approach, fusing a TAM receptor intracellular kinase domain to an extracellular receptor kinase domain not normally expressed in the cells being studied. Although the use of chimeric receptors allows for determination of signaling pathways downstream from a single TAM receptor kinase, data from such experiments must be interpreted conservatively, given evidence provided by Fridell *et al.* (1996), suggesting that the extracellular domain impacts downstream signaling. This issue along with inducible expression of TAM receptors in various cell types and unknown variables such as heterodimerization has made characterization of TAM receptor signaling pathways a complex task.

1. Mer SIGNALING

Much of the evidence delineating Mer signaling pathways is provided by studies of chimeric receptors. This approach originated out of necessity as the ligand for Mer was unknown when many of the studies began. Three well-known signaling pathways, those downstream from PI3K/Akt, PLCγ, and MAPK/ERK (Fig. 2), were linked to Mer tyrosine kinase activation by early studies of chimeric Mer receptors expressed in NIH3T3 fibroblasts (Ling and Kung, 1995). In this context, ligand stimulation of Mer kinase led to cellular transformation exemplified by increased proliferation and DNA synthesis. Additional experiments indicated that activation of the MAPK/ERK pathway correlated with activation of Raf and p90RSK kinases as well as phosphorylation of Shc and association of Grb2 with Mer (Ling and Kung, 1995). Later studies identified Gas6 as a ligand for Mer and confirmed that ligand-dependent activation of endogenous Mer stimulates phosphorylation of ERK1/2 (Chen *et al.*, 1997). Phosphorylation and activation of PLCγ may occur through direct binding of one of its SH2 domains to endogenous phospho-Mer (Todt *et al.*, 2004). Similarly, there is evidence to suggest that PI3K may interact with Mer via an SH2 domain (Sen *et al.*, 2007). However, the coimmunopreciptiation experiments of the previous studies do not demonstrate direct binding and it is possible that association of PI3K and PLCγ with Mer is mediated by interaction of Mer tyrosine 872 with additional adapter proteins such as Grb2 (Georgescu *et al.*, 1999).

The ultimate downstream targets of the PI3K/Akt, PLCγ, and MAPK/ERK pathways may differ according to several factors, including cell type and the tissue microenvironment. In some cells, the PI3K/Akt and MAPK/ERK pathways may act in parallel. In leukemia cells, for example, ligand-dependent activation of an EGFR–Mer chimeric receptor stimulated phosphorylation

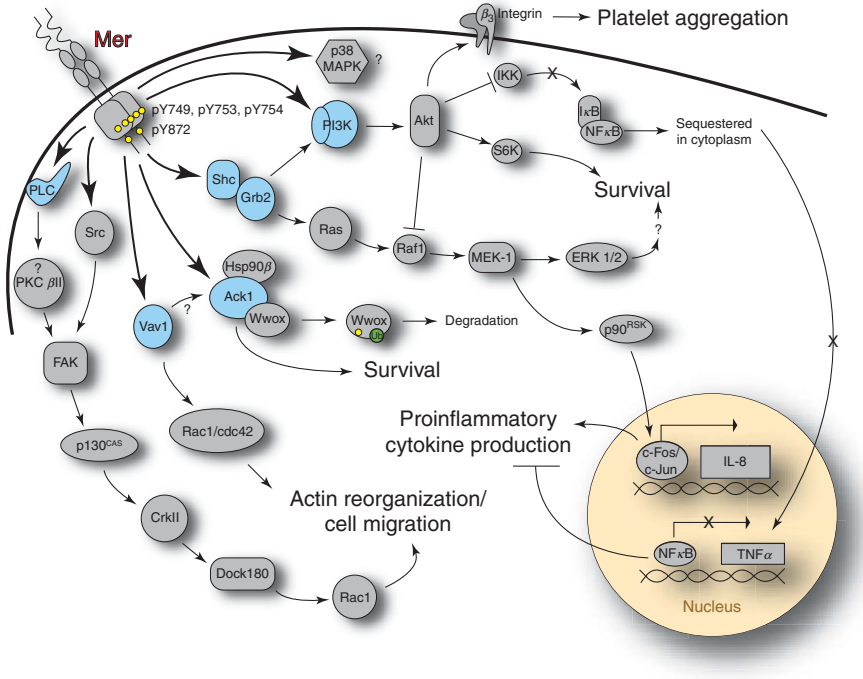

Fig. 2 Mer signaling pathways lead to platelet aggregation, cell survival, regulation of proinflammatory cytokine production, and regulation of the actin cytoskeleton. Molecules in blue have been shown to associate with Mer through either a direct or indirect interaction. Tyrosines 749, 753, and 754 (yellow circles) within the Mer kinase domain are most likely sites of autophosphorylation. Vav1 binds to the region of Mer containing these phosphorylation sites (AA 697–754). It remains undetermined whether the interaction with Mer is direct or mediated by additional adapter proteins. Coimmunoprecipitation experiments suggest that several signaling molecules associate with phosphorylated tyrosine 872 of Mer via their SH2 domains. The kinase(s) which phosphorylate Mer at tyrosine 872 remain unknown. See text for full details. Amino acid designations are from the human sequences. Ub = ubiquitin.

of Akt, ERK1/2, and p38 MAPK resulting in reduced apoptosis without a change in proliferation (Guttridge *et al.*, 2002). The presence of multiple Mer signaling pathways which converge on the same prosurvival outcome gives these cells a strong advantage over noncancerous lymphocytes.

In other instances, the PI3K/Akt and MEK/Erk pathways may act in opposition. Similar to the study of leukemia cells discussed earlier, the PI3K/Akt and MAPK/ERK pathways were activated by ligand stimulation of an FMS–Mer chimeric receptor in prostate cancer cells. Additional experiments demonstrated that the Raf and p90RSK kinases act upstream and downstream, respectively, of MAPK/ERK, leading to transcriptional activation of IL-8 via c-Fos/c-Jun binding to the AP-1 promoter region

(Wu *et al.*, 2004). Preincubation with a MEK inhibitor produced the expected result of decreased IL-8 production. However, preincubation with a PI3K inhibitor increased IL-8 production. The authors therefore speculated that the PI3K/Akt pathway may attenuate the effects of the MAPK/ERK pathway by phosphorylating and inhibiting Raf. In this case, activation of Mer may both stimulate and reduce proinflammatory cytokine production. It should be noted that other studies have suggested that Mer reduces production of proinflammatory cytokines in noncancerous cells (Camenisch *et al.*, 1999; Sen *et al.*, 2007). Ectopic expression of Mer in prostate cancer cells may therefore result in activation of altered downstream signaling pathways. The tonic strength of normal versus aberrant signaling may therefore determine the oncogenic potential of Mer activation and the ultimate phenotypic fate of the tissue.

Yet another possibility exists whereby activation of Mer stimulates a unique complement of signaling events under specific conditions, thus altering the downstream effect(s) of each individual pathway. For example, some studies of Mer signaling suggest that the PI3K/Akt pathway activates NFκB while others suggest that NFκB is inhibited by the PI3K/Akt pathway. Expression of a constitutively active CD8–Mer chimera in pro-B cells resulted in transcriptional activation of NFκB via PI3K/Akt (Georgescu *et al.*, 1999). Additional signaling pathways activated by CD8–Mer included p38/MAPK and MEK1. These cells were protected from apoptosis and became IL-3-independent. Conversely, pretreatment of dendritic cells with apoptotic cells prior to LPS exposure induces Mer-mediated stimulation of PI3K/Akt. Under these experimental conditions, the p38/MAPK, MEK1, and JNK signaling pathways were active but unaffected by Mer stimulation. The phenotypic result in this case was reduced production of the proinflammatory cytokine, TNFα, following exposure to LPS (Sen *et al.*, 2007). Additional experiments in the same study demonstrated that PI3K/Akt negatively regulates NFκB by inhibiting IKK activity and thus preventing degradation of IκB. As is observed with Axl-mediated survival (explained later), PI3K/Akt is classically thought to phosphorylate and activate IκB kinase (IKK), leading to phosphorylation and degradation of inhibitor of κB (IκB) releasing NFκB from the inhibitory complex. However, different isoforms of IKK have been discovered that are differentially phosphorylated by Akt (Gustin *et al.*, 2004). Thus, there are many factors that define the downstream effects of TAM signaling pathways, including the isoforms of numerous kinases involved and the concomitant activity of additional signaling pathways. Clearly, further investigation is needed to elucidate the myriad signaling pathways activated by Mer kinase.

In addition to the well-known pathways mediated by PI3K/Akt, PLCγ, and MAPK/ERK, some atypical signaling pathways have been proposed as a link between Mer and the actin cytoskeleton. Yeast two-hybrid experiments

revealed Mer interactions with Grb2, SHC, and Vav1, the latter is a guanine nucleotide-exchange factor regulating Rac and cdc42 GDP to GTP exchange. Surprisingly, the Mer interaction with Vav1 involved the Vav1 SH2 domain but was constitutive and phosphotyrosine-independent (Mahajan and Earp, 2003). Subsequent Mer activation induced both Vav1 tyrosine phosphorylation and release of Vav1 from Mer. GDP/GTP exchange on Rac1 and cdc42 followed. These small G proteins are commonly recognized as regulators of the actin cytoskeleton. The initial experiments cited earlier were conducted using an EGFR–Mer chimera expressed in 32D cells. Further study, however, demonstrated that Gas6 stimulation of endogenous Mer in human macrophages also results in Vav1 release and subsequent Rac1 and cdc42 GTP loading (Mahajan and Earp, 2003). These data suggest a potential mechanism whereby activation of Mer may induce spatially focused regulation of the actin cytoskeleton, thus providing a model whereby Mer may mediate changes in cellular morphology necessary for phagocytosis of apoptotic cells bound at specific sites on the macrophage surface. Interestingly, the site of Vav1 interaction was mapped to amino acids 697–754 of Mer. This region contains the three putative Mer autophosphorylation sites (see Section II.D.1). As tyrosine phosphorylation of Vav1 was not sufficient for release from Mer, it is enticing to speculate that another SH2 domain-containing protein, perhaps with higher affinity for phosphorylated Mer, is required to release Vav1 and initiate cytoskeletal rearrangement. However, to our knowledge no other proteins have been suggested to interact with Mer in this region.

Another study suggests that Mer regulates the actin cytoskeleton via PLCγ2 and Src. Upon exposure of macrophages to apoptotic cells, PLCγ2 associates with Mer and becomes phosphorylated (Todt *et al*., 2004). PLC can activate classical protein kinase Cs (PKCs) such as PKC βII, which is required for PS receptor-dependent phagocytosis in macrophages (Todt *et al*., 2002). In addition, the Gas6–Mer system may also cooperate with the soluble bridging molecule milk fat globule-EGF factor 8 protein (MFG-E8) and its receptor $\alpha v\beta 5$ integrin to stimulate the lamellipodia formation necessary for phagocytic engulfment of apoptotic cells. Studies utilizing constitutively active Mer chimera and kinase dead mutant Mer demonstrated that Mer stimulates Src-mediated phosphorylation of FAK and $p130^{CAS}$/CrkII/Dock180 complex activation of Rac1 in an $\alpha v\beta 5$ integrin-dependent manner (Wu *et al*., 2005). This pathway may also involve PLCγ2 as FAK association with $\alpha v\beta 5$ integrin is dependent on PKC (Lewis *et al*., 1996a).

Mer activation has also been linked to cell survival via atypical signaling pathways. Gas6 stimulation of a human prostate adenocarcinoma cell line resulted in phosphorylation of a 120-kDa protein that was identified as Cdc42-associated kinase (Ack1) by mass spectrometry (Mahajan *et al*.,

2005). Constitutive association of Mer and Ack1 could be detected by coimmunoprecipitation of the endogenous proteins. Experiments with constitutively active and kinase dead mutant constructs of Ack1 demonstrated that Ack1 is not a direct Mer substrate, but that Ack1 autophosphorylation (and presumably activation) is facilitated by ligand activation of cell surface Mer. Continued Ack1 kinase activity required the chaperone activity of heat shock protein 90β (Hsp90β). Additional mass spectrometry sequencing of constitutively active Ack immunoprecipitates identified the tumor suppressor Wwox as an Ack1-interacting protein. Further investigation suggests that Ack1 induces phosphorylation, ubiquitination, and degradation of Wwox. Downregulation of this proapoptotic tumor suppressor may be one mechanism by which Ack1 and perhaps Mer relay survival signals in cancer cells. Since the physiologic function of the high levels of Mer expressed in normal prostate is not known, it is difficult to assess the normal role of the Mer–Ack axis.

2. Axl SIGNALING

Gas6/Axl signaling promotes the growth and survival of numerous cell types, including cardiac fibroblasts (Stenhoff *et al.*, 2004). These effects are likely mediated by Gas6/Axl-induced activation of the MAPK/ERK and PI3K signaling pathways (Fig. 3). Early studies utilized a chimeric EGFR/Axl receptor expressed in a leukemic cell line. These experiments demonstrated that ligand stimulation of the chimeric receptor leads to cell proliferation via activation of Grb2, Ras, Raf1, MEK-1, and ERK1/2 (Fridell *et al.*, 1996). Interestingly, Grb2 can be activated either by direct binding to tyrosine 821 on Axl or by association with Shc, which is phosphorylated upon ligand stimulation but does not associate with Axl. Later studies confirmed that the Ras/ERK pathway is essential for Gas6-induced mitogenesis of NIH3T3 cells (Goruppi *et al.*, 1999). Importantly, NIH3T3 cells also express Tyro-3 and therefore this mitogenic pathway may be activated by multiple TAM receptors. Although more than one study has suggested that weak or partial activation of the Ras/ERK pathway contributes to Axl-mediated survival (Bellosta *et al.*, 1997; Fridell *et al.*, 1996), more recent data indicate that Ras is dispensable for survival resulting from Gas6 stimulation of native TAM receptors in NIH3T3 cells (Goruppi *et al.*, 1999). However, the MAPK/ERK pathway may be important for Gas6/TAM receptor-mediated survival in certain cell types, including GnRH neurons (Allen *et al.*, 1999).

While the MAPK/ERK pathway typically results in Axl-mediated proliferation, Axl binding to and activation of PI3K has been linked to multiple downstream pathways converging on increased cell survival. One pathway involves classical PI3K stimulation of Akt and S6K (Goruppi *et al.*, 1997).

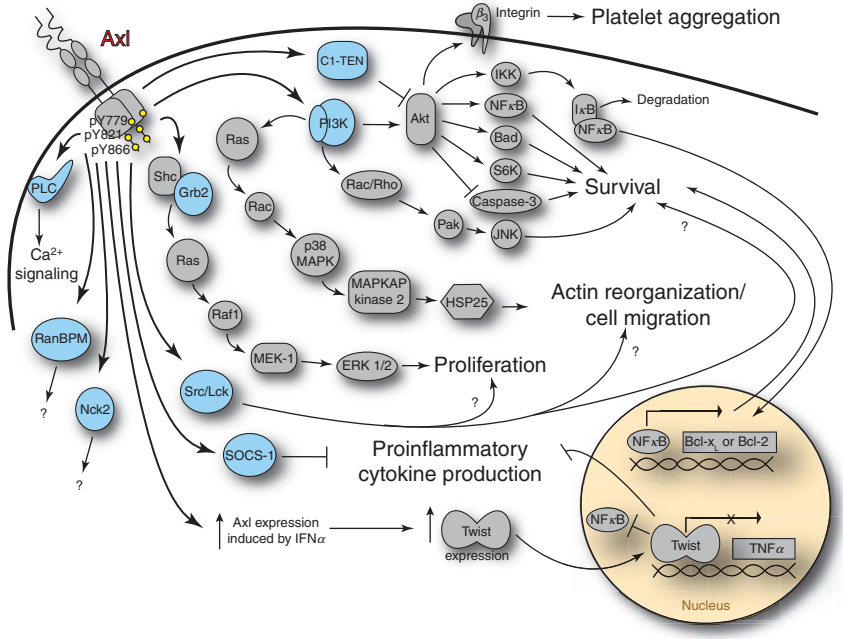

Fig. 3 Axl signaling pathways lead to platelet aggregation, cell survival, proliferation, regulation of proinflammatory cytokine production, and regulation of the actin cytoskeleton. Molecules in blue have been shown to associate with Axl through either a direct or indirect interaction. Tyrosines 779, 821, and 866 of Axl are phosphorylated (yellow circles) and mediate interactions with a number of signaling molecules. It remains unknown whether these residues are sites of autophosphorylation or whether they are substrates for another protein tyrosine kinase. See text for full details. Amino acid designations are from the human sequences.

Gas6 also stimulates phosphorylation of Bad, a target of Akt commonly associated with prosurvival signaling (Goruppi *et al.*, 1999; Lee *et al.*, 2002). Other survival pathways downstream of Gas6–Axl signaling via PI3K/Akt include phosphorylation of NFκB, increased expression of antiapoptotic proteins such as Bcl-2 and Bcl-x_L, and inhibition of proapoptotic proteins such as caspase 3 (Demarchi *et al.*, 2001; Hasanbasic *et al.*, 2004). Transcriptional activation of Bcl-x_L occurs via the cannonical NFκB activation pathway whereby Akt phosphorylates and activates IKK, leading to phosphorylation and degradation of IκB releasing NFκB from the inhibitory complex (Demarchi *et al.*, 2001). NFκB then enters the nucleus where it binds to the promoter region of Bcl-x_L. Interestingly, this mechanism of NFκB regulation by Axl/PI3K/Akt differs from Mer activation of PI3K/Akt, which has been shown to inhibit IKK resulting in downregulation of NFκB-dependent transcription of TNFα (explained later). Another

Gas6/Axl-induced survival pathway may involve PI3K activation of the small GTPases Rac and Rho as well as the downstream kinases Pak and JNK (Goruppi *et al.*, 1999). Many of these experiments were conducted in NIH3T3 cells which express both Axl and Tyro-3. However, Gas6 stimulation of fibroblasts from Axl−/− mice did not result in increased cell survival relative to Axl WT cells (Bellosta *et al.*, 1997). These results suggest that Axl is required for Gas6-mediated survival in some cell types. Additional studies suggest that Gas6/Axl receptor signaling activates PI3K-dependent survival pathways in numerous other cells types, including lens epithelial cells, vascular smooth muscle cells, GnRH neurons, and oligodendrocytes (Allen *et al.*, 1999; Melaragno *et al.*, 2004; Shankar *et al.*, 2003; Valverde *et al.*, 2004). Further study in oligodendrocytes from WT, Axl−/−, and Tyro-3−/− mice suggest that Axl is required for Gas6–PI3K–Akt-mediated survival (Shankar *et al.*, 2006).

In addition to the prototypic growth and survival pathways described earlier, Gas6/Axl signaling has also been linked to additional cellular functions such as neuronal cell migration and cytokine production. Studies of GnRH neurons suggest that Axl directs migration of these cells from the olfactory placode to the forebrain via a signaling pathway involving PI3K, Ras, Rac, p38 MAPK, MAPKAP kinase 2, and HSP25, which results in actin reorganization (Allen *et al.*, 2002; Nielsen-Preiss *et al.*, 2007). Interestingly, Axl is not expressed in postmigratory GnRH neurons (Allen *et al.*, 1999). With respect to cytokine production, IFNα-induced upregulation of Axl and Gas6 expression in human macrophages leads to increased Twist expression and reduced TNFα production (Sharif *et al.*, 2006). Twist is a basic helix loop helix protein that likely inhibits NFκB-mediated transcription of TNFα by binding to the E box region within the TNFα promoter. Given that macrophages also express Tyro-3 and Mer, these receptors may also regulate Twist expression. Consistent with this idea, Protein S (which has not been shown to activate Axl) stimulated Twist expression in the presence of IFNα.

A number of studies have suggested a physical association between Axl and various signaling molecules. For example, coimmunoprecipitation experiments demonstrated association of EGFR/Axl chimera and several coexpressed GST fusion proteins in 293 cells. In the same study, Far–Western analysis of mutant EGFR/Axl receptors as well as competition assays with phosphorylated Axl peptides revealed that tyrosine 821 of Axl mediates binding to PLCγ, p85α and p85β subunits of PI3K, Grb2, Src, and Lck (Braunger *et al.*, 1997). Axl tyrosine 866 also contributes to PLCγ binding while tyrosine 779 may constitute a nonessential, low affinity site of interaction with p85α and p85β. The interaction of Src and Lck likely involves additional contacts *in vivo* as the Axl mutant receptor Y821F effectively coimmunoprecipitated both SFKs from 293 cells. Yeast two-hybrid experiments confirmed the interaction of Axl with PI3K and Grb2 while identifying four novel proteins which

potentially interact with Axl: suppressor of cytokine signaling (SOCS)-1, Nck2, Ran-binding protein in microtubule organizing center (RanBPM), and C1-TEN (Hafizi *et al.*, 2002).

In many cases, such as the Grb2 and PI3K pathways described earlier, the signaling events downstream of these interactions have been subject to intense investigation. Conversely, Src-family kinase activity has been associated with Gas6-mediated mechanisms of proliferation and survival as well as neuronal migration, but the upstream and downstream components of these signaling pathway(s) have not been determined (Goruppi *et al.*, 1997; Nielsen-Preiss *et al.*, 2007). Many of the other Axl-interacting proteins have not been studied beyond their association with activated receptor. Nonetheless, there are reasonable hypotheses as to how some of these proteins may be involved in TAM receptor signaling. C1-TEN, for example, contains a tyrosine phosphatase motif. Thus Axl and other TAM receptors may be found in complex with both tyrosine kinases (SFKs) and phosphatases. Overexpression of C1-TEN in 293 cells has been shown to inhibit Akt signaling resulting in reduced cell survival, migration, and proliferation (Hafizi *et al.*, 2005b). These data are consistent with Axl inactivation mediated by the putative phosphatase C1-TEN. Furthermore, Axl signaling has been associated with attenuation of cytokine production (see Section II.E.1.b), including attenuation of proinflammatory cytokine production following exposure to LPS, a potential role for Axl SOCS-1 signaling as SOCS-1 is implicated in negative regulation of LPS-induced signaling (Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002).

3. Tyro-3 SIGNALING

The Tyro-3 receptor is the least studied of the TAM receptors and the signaling pathways downstream of Tyro-3 activation are poorly understood. Nonetheless, a handful of studies provided clues as to the molecules which mediate Tyro-3 signaling (Fig. 4). Coimmunoprecipitation of Tyro-3 transiently expressed in COS cells revealed a potential interaction with a phosphorylated SFK (Toshima *et al.*, 1995). Because of cross-reactivity of the antibody used, it remains unknown which SFK(s) (Src, Yes, and/or Fyn) interact with Tyro-3. Importantly, all three of these SFKs are highly expressed in tissues of the central nervous system where they are likely to be found colocalized with Tyro-3. Yeast two-hybrid studies identified a number of proteins that potentially interact with Tyro-3, including RanBPM, protein phosphatase 1 (PP1), and the p85 β-subunit of PI3K (Hafizi *et al.*, 2005a; Lan *et al.*, 2000). Sequencing of the DNAs encoding the interacting proteins demonstrated that PI3K binds Tyro-3 via one of its SH2 domains and the interaction was confirmed *in vitro* and *in vivo* by GST pull-down assay and coimmunoprecipitation, respectively. Furthermore, ligand stimulation of an

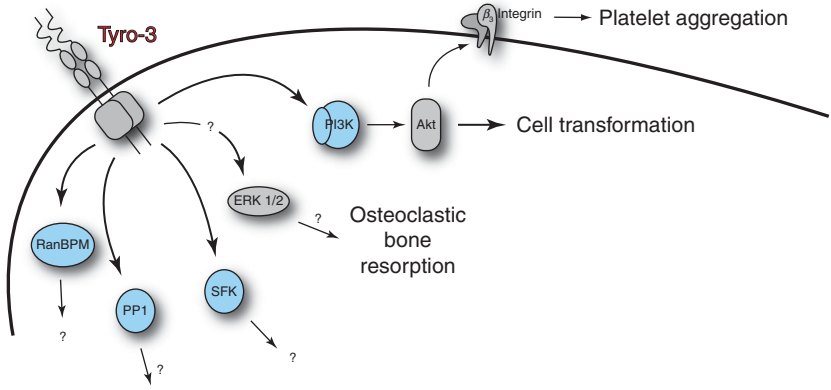

Fig. 4 Tyro-3 signaling pathways mediate platelet aggregation, cell transformation, and osteoclastic bone resorption. Molecules in blue have been shown to associate with Tyro-3 through either a direct or indirect interaction. Phosphorylation of Tyro-3 at specific residues remains uncharacterized.

EGFR/Tyro-3 chimera induces phosphorylation of Tyro-3, PI3K, and Akt resulting in a transformed phenotype. A MAPK signaling pathway has also been linked to Tyro-3 activation as phosphorylation of ERK1/2 was increased by Gas6 stimulation of NIH3T3 cells which express endogenous Tyro-3 (Chen *et al.*, 1997). Phosphorylation of ERK1/2 was also upregulated by Gas6 stimulation of endogenous Tyro-3 in mouse osteoclasts, resulting in bone resorption (Katagiri *et al.*, 2001). Importantly, phosphorylation of Tyro-3 at specific residues has not been described. Clearly, further investigation is necessary to elucidate the signaling pathways downstream of Tyro-3 activation.

III. INVOLVEMENT OF TAM RECEPTORS IN CANCER

There are many ways that protooncogenes such as TAM receptors can be activated, including gene amplification and mutations, proteolytic cleavage, and altered protein expression. These modifications have all been described for TAM receptors and each may result in generation of a constitutively active enzyme and/or over- or ectopically expressed proteins that are not subject to normal cellular regulation. Most of the TAM receptor gene mutations reported involve Mer and retinal degeneration (D'Cruz *et al.*, 2000; Gal *et al.*, 2000; McHenry *et al.*, 2004; Tada *et al.*, 2006; Tschernutter *et al.*, 2006). To date, no activating TAM receptor human mutations have been associated with development of cancer. Although random

retrovirus-induced mutations of Axl correlated with increased transformation of NIH3T3 cells, gene sequencing revealed that the mutations were silent and overexpression of Axl was determined to be a major contributor to cellular transformation (Burchert *et al*., 1998). This idea is consistent with evidence discussed later, which suggests that the oncogenic potential of TAM receptors is related to aberrant regulation of the same signaling pathways and cellular processes in which these receptors normally play a role.

The oncogenic potential of the TAM receptor kinases was immediately evident as each family member was originally cloned from cancer cells and early studies demonstrated that these RTKs exhibit the ability to transform NIH3T3 fibroblasts and BaF3 lymphocytes *in vitro* (Georgescu *et al*., 1999; Lan *et al*., 2000; Ling and Kung, 1995). Some of the most convincing early evidence, however, comes from studies of the avian ortholog of Mer, Eyk (Jia and Hanafusa, 1994). A truncated version of Eyk containing only the tyrosine kinase domain mediates the transforming ability of the virus RLP30, which causes fibrosarcomas, endotheliomas, and visceral lymphomatosis in chickens (Jia *et al*., 1992). Numerous studies have since used a variety of techniques, including immunohistochemistry, Western blotting, microarrays, RT-PCR, and flow cytometry to demonstrate that TAM receptors are ectopically or overexpressed in a wide array of human cancers. Tyro-3 expression has been associated with acute myeloid leukemia (AML) and multiple myeloma. Altered Axl expression has been reported in lung cancer, uterine cancer, breast cancer, ovarian cancer, gastric cancer, colon cancer, prostate cancer, thyroid cancer, liver cancer, renal cell carcinoma, AML, CML, erythroid leukemia, megakaryocytic leukemia, melanoma, osteosarcoma, and glioblastoma. Aberrant expression of Mer has been linked to B- and T-cell acute lymphoblastic leukemias, mantle cell lymphoma, melanoma, rhabdomyosarcoma, pituitary adenoma, gastric cancer, and prostate cancer (Table II).

Hanahan and Weinberg (2000) have proposed six primary cellular functions as "Hallmarks of Cancer" which normal cells acquire during oncogenesis: self-sufficiency in growth signals, insensitivity to antigrowth signals, limitless replicative potential, tissue invasion and metastasis, sustained angiogenesis, and evasion of apoptosis. In this section we will discuss evidence which suggests that TAM receptors contribute to at least three of these six fundamental mechanisms of malignancy.

A. Migration and Invasion

As discussed in Section II, TAM receptor signaling pathways have been linked to regulation of the actin cytoskeleton. The resultant changes in cellular morphology are likely to contribute to TAM receptor regulation of normal

cellular processes such as platelet spreading and phagocytosis (Angelillo-Scherrer *et al.*, *2005*; Mahajan and Earp, 2003). In glioblastoma cells which express elevated levels of Axl, transfection of a dominant negative Axl (Axl-DN) lacking the kinase domain results in reduced motility, altered morphology characterized by loss of filopodia, and loss of cell-to-cell interactions (Vajkoczy *et al.*, 2006). Conversely, stimulation of an ectopically expressed EGF/Mer chimera in a murine leukemic cell line induces rapid (8–24 h) changes in cell morphology, including cell flattening, extension of dendrite-like processes, and adherence (Guttridge *et al.*, 2002). Thus, ectopic expression or overexpression of TAM receptors and resultant downstream changes in cellular morphology may contribute to mechanisms of oncogenesis.

In addition to kinase-mediated links to the actin cytoskeleton, the extracellular domains of TAM receptors contain adhesion molecule-like motifs suggesting that they may be involved in cell–cell contacts. Overexpression of murine Axl in insect cells results in cell aggregation (Bellosta *et al.*, 1995). *In vitro* experiments with fluorescently labeled Axl-expressing cells and unlabeled Axl-negative cells did not result in mixed aggregates suggesting that the observed cell aggregation is mediated by homophilic binding of Axl receptors on neighboring cells. Additional experiments demonstrated that Axl-expressing cells bind to immobilized Axl extracellular domain (ECD) providing further evidence that Axl ECDs are capable of homophilic binding. In these experiments, Axl–Axl interaction was independent of calcium and Axl kinase activity. Interestingly, overexpression of human Axl in a mammalian leukemia cell line (32D) is not sufficient to induce cell aggregation (McCloskey *et al.*, 1997). Rather, addition of Gas6 is required to induce cell aggregation and this effect is blocked by excess Axl ECD peptide. Experiments with truncated Gas6 demonstrated that either the Gla domain and/or the EGF motifs of Gas6 bind to 32D cells leaving the LG domains of Gas6 available for interaction with Axl. These results suggest that cell aggregation is the result of a Gas6-mediated interaction between Axl and neighboring cells. It is unknown whether phospholipids or another integral membrane protein mediates the interaction of Gas6 with 32D cells. These studies suggest that TAM receptors can mediate cell–cell contacts via receptor–receptor or receptor–ligand interaction. The determinants of adhesion likely involve cell type and tissue microenvironment.

Adhesion molecules are important not only for cell–cell contacts, but also for interaction of cells with their extracellular environment. Axl expression correlates with adherence of lung cancer cell lines (Wimmel *et al.*, 2001). However, forced expression of Axl did not induce an adherent phenotype in small cell lung cancer (SCLC) cells which normally grow in suspension. One interpretation of these data is that Axl expression occurs as a consequence of cellular adhesion rather than playing a causative role in the adherent phenotype. Alternatively, additional factors may be required in order for Axl to

mediate cell adhesion. Axl ligands are the most obvious candidates, although the presence of Gas6 did not induce adherence in the previous experiments and Protein S has not been shown to bind Axl. Another candidate is soluble Axl (sAxl), which is present at high levels *in vivo*. Incubation of Axl-expressing cells with immobilized Axl–Fc induces phosphorylation of Axl and PI3K suggesting that Axl–Fc/Axl interaction activates the kinase domain of the full-length receptor (Budagian *et al*., 2005a). Thus, sAxl may mediate interactions between full-length Axl and the extracellular matrix.

Given that regulation of cell adhesion and morphology are precursors to more complex cellular processes such as cellular migration, the aforementioned studies suggest that both the extracellular and kinase domains of TAM receptors may contribute to oncogenic mechanisms such as cellular migration and tissue invasion. Indeed, Axl has been shown to be involved in normal migration of GnRH neurons from the olfactory placode to the hypothalamus (Allen *et al*., 2002). Interestingly, Axl expression in GnRH neurons is present during migration but then disappears once the cells reach their destination. Aberrant TAM receptor expression could therefore lead to new migratory function and increase invasiveness of cancer cells. In a dorsal skinfold xenograft model, human glioblastoma cells transfected with WT Axl showed significantly greater tumor growth and tissue invasion than cells transfected with truncated Axl lacking the kinase domain (Vajkoczy *et al*., 2006). These results suggest that Axl kinase contributes to tumorigenesis *in vivo*. *In vitro* studies confirmed that reduced migration and invasion resulted from loss of Axl kinase domain and was not an artifact of reduced tumor cell load (Vajkoczy *et al*., 2006). Soluble Axl bound to the extracellular matrix may constitute a chemoattractant for Axl-mediated migration as scratch tests revealed that immobilized Axl–Fc promotes migration of primary fibroblasts prepared from Axl WT mice (Budagian *et al*., 2005a). Primary fibroblasts prepared from Axl−/− mice exhibited reduced migration. Further study is necessary to determine whether increased production of sAxl correlates with tumor invasiveness.

B. Angiogenesis

Formation of new blood vessels is a normal process important during development as well as wound healing. In addition, angiogenesis promotes tumor growth and malignant transformation. Proliferation and migration of vascular smooth muscle cells (VSMCs) are key events required during normal angiogenesis. VSMCs express Gas6 and exogenous application of purified or recombinant Gas6 promotes proliferation and migration of VSMCs (Fridell *et al*., 1998; Nakano *et al*., 1995). Gas6-induced migration of VSMCs was blocked by inclusion of recombinant Axl–ECD. Furthermore,

overexpression of Axl increased migration 2–5-fold whereas expression of a kinase dead mutant reduced migration ~50% relative to parental VSMCs (Fridell *et al.*, 1998). These results demonstrate that migration of VSMCs correlates with the level of Axl kinase activity. It has also been suggested that Axl plays a role in flow-induced vascular remodeling by regulating VSMC apoptosis (Korshunov *et al.*, 2006).

The role of TAM receptors in angiogenesis is not restricted to VSMCs as a genetic screen identified Axl as a regulator of human umbilical vein endothelial cell (HUVECs) migration (Holland *et al.*, 2005). Genetic silencing of Axl or Gas6 significantly reduced migration of HUVECs, whereas overexpression of Axl WT protein increased HUVEC growth and tube formation. Overexpression of a kinase dead Axl mutant, however, reduced HUVEC growth but had no effect on tube formation (Holland *et al.*, 2005). Consistent with these results, glioblastoma xenografts containing a Axl-DN construct exhibited blood vessel density and diameter similar to WT Axl xenografts (Vajkoczy *et al.*, 2006). These results suggest that Axl kinase activity is important for regulation of endothelial cell growth, whereas tube formation is likely regulated by Axl in a kinase-independent manner. Finally, stable shRNA knockdown of Axl reduces blood vessel formation and functional circulation in a mouse model of angiogenesis supporting a role for Axl in angiogenic processes *in vivo* (Holland *et al.*, 2005). Although Tyro-3 and Mer are expressed in endothelial cells (Chan *et al.*, 2000; Sather *et al.*, 2007), their involvement in angiogenesis has not been investigated.

C. Cell Survival and Tumor Growth

Several lines of evidence (see Section II.F) suggest that TAM receptors activate prosurvival signaling pathways in both normal and cancerous cells. In some cases, TAM receptor signaling pathways prevent apoptosis without stimulating proliferation (Guttridge *et al.*, 2002). On the other hand, TAM receptors have also been shown to increase proliferation without inhibiting apoptosis (Sainaghi *et al.*, 2005). A third situation exists, whereby TAM receptors promote both survival and proliferation (van Ginkel *et al.*, 2004). Each mechanism provides a means by which TAM receptors may contribute to tumor growth.

As discussed in Section II.F.1, one mechanism of Mer-mediated cell survival involves activation of Ack1 and subsequent downregulation of the tumor suppressor Wwox (Mahajan *et al.*, 2005). In the same study, expression of constitutively active Ack1 in human prostate adenocarcinoma cells induced anchorage-independent growth *in vitro* and dramatically increased tumor growth in an ectopic xenograft model. Furthermore, patient samples of androgen-independent prostate cancer (AICaP), an advanced stage of prostate

cancer with no curative treatment, exhibited 4–5-fold more phosphorylated (i.e., active) Ack1 and approximately 6-fold less Wwox protein than does normal prostate. These data suggest that Mer stimulation not only activates prosurvival pathways, but also downregulates proapoptotic pathways in this cell type.

Mer is not expressed in normal mouse or human lymphocytes but is ectopically expressed in T-cell leukemias and E2A-PBX1 positive B-cell leukemias (Graham *et al*., 2006; Yeoh *et al*., 2002). To further investigate the oncogenic potential and causal role of Mer in malignancy, our lab developed a Mer transgenic (Mer_{Tg}) mouse model (Keating *et al*., 2006). Using a C57Bl/6 background mouse, the expression of full-length Mer cDNA was introduced under the control of a Vav1 promoter in mouse lymphocytes and thymocytes. Mer_{Tg} lymphocytes exhibited a functional survival advantage *in vitro* compared with wild-type lymphocytes when treated with glucocorticoids, a standard leukemia therapy. The Mer_{Tg} lymphocytes also exhibited phosphorylation of Mer and robust activation of antiapoptotic pathways, including Akt and Erk1/2. Additionally, ectopic expression of Mer in lymphocytes, as is found in T-cell lymphoblastic leukemia patient samples, promoted the development of T-cell predominant leukemia and lymphoma in 55–58% of mice compared to a WT rate of only 12%. Interestingly, Mer expression was significantly correlated with the immature thymocyte stage (CD3, CD4, and CD8 negative), which is a subset of leukemia that portends a poor prognosis with difficulty to reach and retain remission. Resistance to conventional therapies, such as glucocorticoids, in Mer positive leukemias may also indicate a poor prognosis. The Mer transgenic mouse model of T-cell acute lymphoblastic leukemia (T-ALL) therefore suggests that TAM receptors may provide a novel target for future therapy development. Further investigation is necessary to substantiate a correlation between TAM receptor induced cell survival and carcinogenesis and tumor growth *in vivo*.

D. TAM Receptors as Prognostic Factors

Elevated Axl expression correlated with adherence, motility, and invasiveness of osteosarcoma cell lines selected for their high metastatic ability in an *in vivo* model of lung metastasis (Nakano *et al*., 2003). In addition, lung metastasis has been correlated with reduced overall survival of osteosarcoma patients (Tsuchiya *et al*., 2002). The previous results therefore suggest that Axl expression may correlate with poor prognosis in osteosarcoma. Similarly, analysis of 58 adenocarcinoma patient samples revealed that Axl expression significantly correlated with metastatic cancer of advanced clinical stage (Shieh *et al*., 2005). Axl expression also correlated with invasiveness of lung cancer cell lines *in vitro*. In 54 patient samples of AML, Axl

expression correlated with worse progression-free and overall survival (Rochlitz *et al.*, 1999). Interestingly, coexpression of both Mer and Axl correlates with poor prognosis in gastric cancer (Wu *et al.*, 2002), suggesting that cooperativity of multiple TAM receptors may play a role in progression and metastasis of some cancers. These data suggest that TAM receptor signaling may play a role in the progression of multiple cancers, including the development of metastasis.

IV. POTENTIAL THERAPEUTIC APPLICATIONS

Several studies have validated the therapeutic potential of targeting the TAM family in cancer therapy (Table III). Axl RTK and the ligand Gas6 are overexpressed in human glioma cell lines and malignant glioma patient samples when evaluated by microarray, Northern blot, Western blot, and immunohistochemistry analysis (Vajkoczy *et al.*, 2006), leading to baseline constitutive activation of Axl RTK. In order to further investigate the role of Axl in glioblastoma tumorigenesis, these researchers transfected SF126 cells, a human glioma cell line exhibiting high levels of Axl RTK expression, with either wild-type Axl (Axl-WT) or Axl-DN which lacks the tyrosine kinase domain. Cells expressing Axl-DN demonstrated inhibition of Axl RTK activity, decreased proliferation, and reduced invasive potential relative to Axl-WT. Orthotopic implantation of Axl-DN cells resulted in markedly reduced tumor growth *in vivo* compared to Axl-WT. Furthermore, mice which received Axl-WT died within 30 days of implantation whereas 50% of the mice which received Axl-DN were still alive 70 days after

Table III Axl as a Therapeutic Target

Inhibitor	Outcome	Reference
Axl-DN construct	Reduced glioblastoma growth and invasiveness *in vitro* and *in vivo*; Increased overall survival after orthotopic implantation of glioblastoma cells containing Axl-DN	Vajkoczy *et al.* (2006)
shRNA Axl construct	Decreased growth of breast carcinoma tumors in an ectopic xenograft model	Holland *et al.* (2005)
Axl small molecule inhibitor (MP470)	May inhibit *in vitro* Axl kinase activity with limited selectivity; cytotoxic to gastrointestinal stromal tumor cells *in vitro*	Mahadevan *et al.* (2007)

implantation. This study suggests that inhibition of Axl kinase activity reduces glioblastoma tumor growth and invasiveness and improves overall survival.

Axl RTK is expressed in lung adenocarcinoma cell lines, and the level of Axl expression correlates with the invasive ability of these cell lines *in vitro* (Lay *et al*., 2007; Shieh *et al*., 2005). Ectopic overexpression of Axl in adenocarcinoma cell lines leads to increased formation of filipodia, migration, and drug resistance. Conversely, shRNA knockdown of Axl protein levels results in decreased migration (Lay *et al*., 2007). These studies suggest that blockade of Axl signaling would offer a new therapeutic strategy for this tumor type.

Overexpression of TAM receptors has also been reported in breast cancer (Berclaz *et al*., 2001; Taylor *et al*., 1995b). Stable shRNA knockdown of Axl significantly reduced tumor growth in a xenograft model of breast carcinoma (Holland *et al*., 2005). In the same study, inhibition of Axl with small interfering RNA in human umbilical vein endothelial cells (HUVECs) blocked endothelial tube formation *in vitro* suggesting that inhibition of Axl may restrict mechanisms of angiogenesis required for breast cancer tumor cell growth. The aforementioned studies suggest that downregulation of Axl and its family members with currently existing or new biologically targeted tyrosine kinase inhibitors may prove to be a viable treatment option for historically difficult to treat cancers such as glioblastoma and drug-resistant lung adenocarcinoma.

Because the TAM family of tyrosine kinases has been implicated in the pathophysiology of several malignancies, they offer unique targets for new therapeutics. Several nonspecific tyrosine kinase inhibitors (e.g., Gleevec, Erlotinib, Dasatinib, and others) are already in use for a variety of malignancies, and have proven to be both efficacious and less toxic than standard chemotherapies. These tyrosine kinase inhibitors may prevent activation of the TAM family kinases in addition to other RTKs, leading to downregulation of cell survival pathways, thereby slowing growth and metastasis of malignancy. Additional mechanisms of TAM receptor inhibition could include soluble receptors that soak up available ligand, or direct binding to the receptor by monoclonal antibodies. The latter might block activation, desensitize, or downregulate the surface receptor, or call in an immune response. Many of these types of activities have been ascribed to the anti-HER2 monoclonal antibody, Herceptin.

A. Small Molecule Inhibitors

To date, only one small molecule inhibitor designed to inhibit TAM receptor function has been reported in the literature. MP470 is a potential Axl inhibitor but also blocks other tyrosine kinases within the same

concentration range (Mahadevan *et al.*, 2007). Sensitivity of Mer and Tyro-3 to MP470 has not been tested. Thus, it is not clear how selectively this molecule inhibits Axl. Nevertheless, MP470 reduces the metabolic activity of an Axl-expressing, drug-resistant, gastrointestinal stromal tumor (GIST) cell line, suggesting that this novel drug may provide new treatment strategies for drug-resistant cancers. In addition, these studies further validate Axl as a therapeutic target for treatment of cancer and provide promising evidence for future selective small molecule inhibitors of TAM receptors.

B. Soluble Receptors

As discussed previously in Section II.D.3, the TAM family members undergo alternative splicing or shedding or both of their extracellular portion, leading to production of soluble receptors. These soluble receptors lack the tyrosine kinase domain, and act as a ligand sink to sequester ligand, thereby limiting signaling through the full-length RTK (Costa *et al.*, 1996). For example, treatment of NIH3T3 cells with soluble Axl ectodomains led to inhibition of Axl signaling and a decrease in DNA synthesis (Costa *et al.*, 1996).

In investigating soluble ectodomains as a potential treatment mechanism in malignancy, Sainaghi *et al.* (2005) evaluated proliferation of Axl-expressing prostate carcinoma cell lines. They found that treatment with secreted Axl ectodomains abrogated Gas6-induced stimulation and cell proliferation.

C. Antibodies

Antibody therapy for cancer treatment has been theoretically promising but only successful in limited areas, such as Rituximab (anti-CD20) used in treating lymphoma (Foran *et al.*, 2000a,b) or anti-GD2 used in neuroblastoma (Handgretinger *et al.*, 1992, 1995). The discovery of novel important targets in the oncogenic process that can be effectively inhibited by antibody presence has been the limiting step. The ectopic surface expression of TAM family members, such as occurs with Mer in lymphoblastic leukemia and lymphoma, makes inhibition of TAM receptors with antibodies attractive.

Angelillo-Scherrer *et al.* (2001) have provided *in vivo* proof of concept of inhibition of TAM receptors with an anti-Gas6 antibody to prevent fatal thrombosis in mouse models. Therefore, in the case of cancer therapeutics this antibody could potentially be used to neutralize Gas6 activity and thus reduce signaling through all of the TAM family members. Further investigations into antibody use and application are warranted.

D. Liabilities of TAM Receptor Antagonism

Because the TAM family of RTKs performs several normal cellular functions (see Section II.E), there is potential for concern regarding the effects of inhibiting these RTKs. For example, mutations in the Mer gene lead to defective phagocytosis of photoreceptor outer segments by the RPE resulting in retinal degeneration. However, rodent studies suggest that retinal degeneration only occurs after prolonged Mer inhibition. These data are supported by reports of human patients with deactivating mutations of Mer (Gal *et al.*, 2000). In three patients described with either heterozygous or homozygous Mer mutations, poor vision and night blindness were first noted in childhood. Thus retinal degeneration caused by Mer inhibition is likely to develop gradually over the course of several years and there may be a "therapeutic window" during which short-term therapy with agents biologically targeted to inhibit Mer may be a feasible strategy for treatment of Mer positive cancers such as lymphoblastic leukemia. Furthermore, if vision changes were observed, studies in rodents suggest that cessation of therapy would restore normal vision (Vollrath *et al.*, 2001).

While the above example is specific to Mer antagonism, inhibition of Axl and Tyro-3 also elicit potential concerns. As discussed in Section II.E.1, TAM knockout mice develop autoimmune diseases including lupus-like syndrome (Cohen *et al.*, 2002; Lemke and Lu, 2003). In a similar manner, chronic antagonism of TAM receptors may lead to autoimmune disease in humans. However, such effects are unlikely with short-term therapeutic inhibition of TAM receptors. Furthermore, autoimmunity phenotypes were most pronounced in double and triple TAM receptor knockouts suggesting that the development of biological therapeutics which selectively target individual TAM family members would reduce the likelihood of adverse effects.

Conventional chemotherapies cause a multitude of serious toxicities, most notably bone marrow suppression, kidney and liver dysfunction, and neuropathies. There is no evidence to suggest that TAM RTK inhibition would have overlapping toxicity profiles with current conventional therapies. In fact, one might expect that selective inhibitors of TAM receptors would exhibit minimal systemic toxicity. Nevertheless, this is an area that requires further preclinical investigation. Certainly the safety of many nonspecific tyrosine kinase inhibitors is already well proven. It remains to be seen whether these inhibitors affect the TAM RTK family, thereby inhibiting cell survival pathways as is hypothesized. More direct and specific inhibition of Mer, Axl, and Tyro-3 using selective small molecule tyrosine kinase inhibitors, soluble receptors, and/or antibodies has not been accomplished in animal models of cancer as yet, and therefore more research is needed.

V. CONCLUSIONS

In the last decade, research has established the link between abnormal TAM receptor expression and oncogenesis. All three receptors are over- or ectopically expressed in a wide spectrum of human cancers, and overexpression of TAM receptors is sufficient to transform cells. TAM receptor inhibitions in animal xenograft tumor models of glioblastoma and breast cancer have provided preliminary validation of this receptor family as a cancer therapy target.

More than half of the known RTKs have been directly implicated in human cancer. Although cancer is a multistep process, the success of targeted RTK inhibition in clinical cancer trials has demonstrated that blocking activity of a single dominant activated RTK can affect tumor growth, leading to widespread development of this class of drugs. In fact, in the last 15 years, novel targeted therapies led to the FDA approval of more cancer drugs than in the preceding 40 years combined. Some of the targeted therapies against tyrosine kinases, such as inhibitors of abl, EGFR, and VEGFR, have clearly improved patient survival with minimal additional toxicity. Many posit that this phase of tyrosine kinase inhibitory drug development is winding down. But as the ability to molecularly type human tumors and uncover or implicate additional tyrosine kinases as targets in tumor subsets improves, we believe this group of targets and their inhibitors will continue to supplement cytotoxic therapy.

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