Published in final edited form as:
Cell. 2014 August 28; 158(5): 1033–1044. doi:10.1016/j.cell.2014.06.048.

A SECRETED TYROSINE KINASE ACTS IN THE EXTRACELLULAR ENVIRONMENT

Mattia R. Bordoli[1], **Jina Yum**[1,7], **Susanne B. Breitkopf**[2,3], **Jonathan N. Thon**[4,5], **Joseph E. Italiano Jr**[4,5,6], **Junyu Xiao**[8], **Carolyn Worby**[8], **Swee-Kee Wong**[1], **Grace Lin**[1], **Maja Edenius**[1], **Tracy L. Keller**[1], **John M. Asara**[2,3], **Jack E. Dixon**[8], **Chang-Yeol Yeo**[1,7], and **Malcolm Whitman**[1]

[1]Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, USA

[2]Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, MA 02115, USA

[3]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

[4]Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, USA

[5]Harvard Medical School, Boston, MA 02115, USA

[6]Vascular Biology Program, Department of Surgery, Children's Hospital, Boston, MA 02115, USA

[7]Department of Life Science & Global Top5 Research Program, Ewha Womans University, Seoul 120-750, Republic of Korea

[8]Department of Pharmacology, University of California - San Diego, 92031 La Jolla, CA USA

Summary

Although tyrosine phosphorylation of extracellular proteins has been reported to occur extensively in vivo, no secreted protein tyrosine kinase has been identified. As a result, investigation of the potential role of extracellular tyrosine phosphorylation in physiological and pathological tissue regulation has not been possible. Here we show that VLK, a putative protein kinase previously shown to be essential in embryonic development, is a secreted protein kinase, with preference for tyrosine, that phosphorylates a broad range of secreted and ER-resident substrate proteins. We find that VLK is rapidly and quantitatively secreted from platelets in response to stimuli, and can tyrosine phosphorylate co-released proteins utilizing endogenous as well as exogenous ATP sources. We propose that discovery of VLK activity provides an explanation for the extensive and conserved pattern of extracellular tyrosine phosphophorylation seen in vivo, and extends the importance of regulated tyrosine phosphorylation into the extracellular environment.

Correspondence to: Malcolm Whitman mwhitman@hms.harvard.edu, Chang-Yeol Yeo, cyeo@ewha.ac.kr 617 432 1320. Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, US.

Introduction

An extraordinary and little-recognized observation has emerged from analysis of the mammalian phosphoproteome: a substantial portion of secreted or extracellular proteins have been reported to contain phosphotyrosine (Hornbeck et al., 2004), but, to date, no secreted tyrosine kinase has been identified. Tyrosine phosphorylation of a wide range of extracellular matrix (ECM) and pericellular proteins has been reported in high throughput liquid chromatography tandem mass spectrometry (LC MS/MS) analysis of tissue samples of both healthy and diseased tissues (Hornbeck et al., 2004). In many instances, tyrosine phosphorylation at these sites has been reported in multiple independent studies using material from both cultured cell and primary tissue samples (Ballif et al., 2008; Hornbeck et al., 2004). In light of the emerging importance of ECM as a dynamically regulated microenvironment with a fundamental role (Hynes and Naba, 2011; Lu et al., 2011) in the control of cell differentiation, survival, and function, tyrosine phosphorylation of ECM proteins represents a potential major new mechanism of tissue regulation.

Phosphorylation of the secreted acidic milk protein casein has been known for over a century (Tagliabracci et al., 2013; Yalak and Vogel, 2012), but only in 2012 was the kinase responsible for the serine phosphorylation of casein and other acidic secreted proteins identified (Tagliabracci et al., 2012). Fam20C, a Golgi localized protein kinase, is a serine kinase that phosphorylates both casein and other highly acidic proteins such as osteopontin and members of the small integrin-binding ligand, N-linked glycoproteins (SIBLING) family at the target motif SerXGlu (Tagliabracci et al., 2012). Phosphorylation by Fam20C in the secretory pathway is robust, and essential for proper biomineralization of bone. The substrate specificity of FAM20C indicates, however, that it is not likely to account for the tyrosine phosphorylation of the secreted proteins described above. The discovery of FAM20C highlighted, however, that the mammalian secretory kinome may be largely unexplored, with an unknown number of secreted protein kinases yet to be identified (Tagliabracci et al., 2013; Yalak and Vogel, 2012).

In many ECM and matricellular proteins, tyrosine phosphorylations occur at structurally conserved positions in otherwise distantly related proteins (Hornbeck et al., 2004), suggesting a conserved regulatory roles for these modifications The hemopexin domain of matrix metalloproteinases (MMPs) provides a clear example of this phenomenon. Seven different MMP family members have been reported to be tyrosine phosphorylated within their C-terminal hemopexin domains (Figure 1A) (Guo et al., 2008; Hornbeck et al., 2004; Raijmakers et al., 2010; Rikova et al., 2007). In each case, the phosphotyrosine is found at a conserved β-bulge in blade 2 of the hemopexin 4-blade propeller structure (Fig. 1B). Strikingly, phosphorylation at an identical position is also seen in lubricin (Rinschen et al., 2010) and hemopexin (Rinschen et al., 2010), proteins which contain the hemopexin domain structure, but which are only distantly related to MMPs (Fig. 1B). While the structural conservation of these tyrosine phosphorylations is intriguing, in the absence of any known secreted tyrosine kinases there has been no way to study the regulation or role of this novel class of tyrosine phosphorylations.

To understand the regulation and significance of the tyrosine phosphorylation of secreted proteins, we re-examined the existing kinome and associated literature for unrecognized secreted protein kinases. The putative protein kinase VLK/PKDCC (hereafter VLK) emerged from this search as a promising candidate for a secreted tyrosine kinase. The VLK gene (also known as SGK493, ADTK1, and AW548124) was cloned as a putative protein kinase expressed in differentiating embryonic stem cells (Goncalves et al., 2011; Imuta et al., 2009; Kinoshita et al., 2009). $VLK^{-/-}$ mice die within one day of birth, with severe defects in morphogenesis in multiple tissues, indicating that VLK is essential for patterning at organogenesis stages (Goncalves et al., 2011; Imuta et al., 2009; Kinoshita et al., 2009; Probst et al., 2013). VLK encodes a protein with clear homology to protein kinases (Fig. 2A, Fig S2), but lacking recognizable homology in kinase subdomains V, IX, X, and X1 (Hanks, 2003; Kinoshita et al., 2009), and by primary sequence is not distinguishable as a member of the major serine-threonine, tyrosine, or dual specificity subsets of intracellular protein kinases (Fig. S2A). Strikingly, VLK contains a predicted hydrophobic sequence near the N-terminus that we find to be recognized as a signal sequence by multiple signal sequence prediction algorithms (Horton et al., 2007; Petersen et al., 2011) (Figure 2A). Both the kinase domain and the predicted signal peptide are conserved across vertebrates (Figure S2B). Early studies of VLK suggested that it might autophosphorylate on tyrosine, but no kinase activity of VLK towards exogenous substrates was seen (Kinoshita et al., 2009). The predicted signal peptide in VLK, and the indirect evidence for VLK autophosphorylation on tyrosine, however, suggested VLK as a strong candidate as a kinase to explain the tyrosine phosphorylations in secreted proteins reported in Phosphosite.

We demonstrate here that VLK is a secreted protein kinase with preference for tyrosine residues, and that it phosphorylates secreted proteins at sites identical to many of those previously reported to occur in vivo. Expression of VLK results in the tyrosine phosphorylation of both secreted and ER/Golgi resident proteins, and these phosphorylations are dependent on the signal peptide as well as the active kinase domain of VLK. VLK is constitutively secreted by mammalian cell lines, and is highly expressed in human platelets, localized to secretory granules, and quantitatively released following platelet degranulation. Furthermore, tyrosine phosphorylation of proteins secreted from stimulated platelets is VLK dependent, and can be supported by endogenous secreted ATP. These observations identify VLK as the first secreted protein tyrosine kinase, which can act not only in the secretory pathway but also following its regulated release outside the cell.

RESULTS

VLK localizes to the secretory pathway and is glycosylated

To test localization of VLK to the lumenal compartment of the secretory pathway, we expressed VLK in 293T cells and treated detergent free cell lysates with proteinase K (PK) alone or in combination with NP40. Proteins localizing to the cytoplasm, like GFP, are degraded by PK treatment, while proteins localized to the lumenal side of the ER or Golgi are protected by the lumenal membrane in the absence of detergent (Brown et al., 1987). VLK was not degraded by PK alone, but was completely degraded with the addition of NP40 (Figure 2B), indicating that VLK localizes to a membrane protected subcellular

compartment. To test if the predicted N-terminal signal peptide in VLK is responsible for its localization, we generated a tagged construct lacking the first 23 amino-acids (VLK^{-SP}). VLK^{-SP} was completely degraded following PK treatment in the absence of detergent (Figure 2C), demonstrating signal-peptide dependent localization of VLK to the secretory pathway. Furthermore, full-length VLK, but not VLK^{-SP} was found in the conditioned media (CM) of transfected cells (Figure 2D) confirming the requirement of the VLK signal peptide for secretion. Moreover VLK, but not VLK^{-SP}, is strongly phosphorylated on tyrosine (Figure 2D).

VLK contains 5 potential sites for N-linked glycosylation (AsnXSer/Thr). To test if VLK is glycosylated in the secretory pathway, we treated lysates of cells expressing either VLK or VLK^{-SP} with PNGase F, which removes N-linked oligosaccharides. A shift in gel migration was observed for VLK, indicating the presence of N-linked glycosylations, whereas VLK^{-SP} was unaffected (Figure 2E).

Endogenous VLK is secreted into CM

We next examined the localization of endogenous VLK to the secretory pathway. Preliminary screening established detection of endogenous VLK in several established lines, with high expression seen in HepG2 liver hepatoma cells. As for ectopically expressed VLK, endogenous VLK in HepG2 cells was not degraded by PK treatment in absence of NP40 (Figure 2F). Progressive and quantitative accumulation of endogenous VLK was detected in the CM of HepG2 cells (Figure 2G). VLK secretion was abrogated by the addition of the ER/Golgi protein transport inhibitor brefeldin A, with a corresponding increase in cell-associated VLK (Figure 2G). Secreted VLK is found in the supernatant of HepG2 condition medium centrifuged at 100,000 xG, indicating that it is not associated with microvesicular or exosome particles (Figure 2H). The specificity of antibody detection of endogenous VLK was confirmed by lentiviral-mediated knockdown of *Vlk* with two independent shRNA constructs (Figure 2I). These data establish that endogenous VLK is quantitatively localized to the secretory pathway, and is constitutively released from cultured cells.

VLK phosphorylates substrates following stimulated release from platelets

To explore the possibility that VLK secretion is physiologically regulated, we examined the ligand stimulated degranulation of human platelets. VLK is abundantly expressed in platelets (Figure 3A, S3A). Stimulation of platelet degranulation with either the thrombin receptor agonist thrombin receptor activating peptide (TRAP) (Ahn et al., 1997) or phorbol-12-myristate-13-acetate (PMA) (Rozenvayn and Flaumenhaft, 2003), led to the rapid release of VLK into the cell supernatant, concomitant with the release of the alpha granule markers von Willebrand Factor (vWF) protein and thrombospondin (TSP1) (Harrison and Cramer, 1993) (Figure 3A, 3B). Notably, TRAP stimulation resulted in a dramatic decrease in the amount of cell-associated VLK concomitant with the increase of VLK in the cell supernatant, indicating that the stimulus-dependent release of VLK is nearly complete.

Anti-pTyr immunoblotting of unstimulated versus TRAP stimulated platelets revealed that several tyrosine phosphorylated proteins are detectable in the releasate. The most prominent of these secreted phosphotyrosine bands co-migrates with VLK, suggesting that secreted VLK is tyrosine phosphorylated endogenously in platelets (Figure 3A). Addition of supplemental ATP to cell-free supernatant released from TRAP or PMA stimulated platelets resulted in an increase in tyrosine phosphorylation of releasate proteins, indicating that tyrosine phosphorylation can occur in the extracellular environment following regulated tyrosine kinase secretion (Figure 3A). Depletion of secreted VLK with an anti-VLK antibody markedly decreased tyrosine phosphorylation following ATP addition, establishing that secreted VLK phosphorylates substrates in the extracellular environment (Figure 3B). While VLK dependent tyrosine phosphorylation was strongly stimulated by ectopic ATP addition, significant tyrosine phosphorylation of identical bands was seen in the absence of ATP addition (Fig. 3B). Since platelets secrete ATP from dense granules concomitantly with protein release from alpha granules (McNicol and Israels, 1999), we examined the effect of depletion of endogenous co-released ATP with added apyrase on tyrosine phosphorylation. Apyrase addition dramatically reduced phosphorylation of a set of tyrosine phosphoprotein bands similar to those reduced by VLK depletion (Fig. 3B, marked with asterisks), indicating that endogenous ATP can support the tyrosine phosphorylation of protein secreted from platelets subsequent to degranulation (Fig. 3B).

Immunolocalization of VLK in unstimulated platelets by confocal microscopy shows a punctate pattern consistent with localization to alpha granules (Figure 3C, Figure S3B), and electron microscopy confirms VLK localization to these structures (Figure 3D). Immunohistochemical staining of mouse megakaryocytes, which give rise to mature platelets (Italiano et al., 1999), showed a punctate distribution similar to that seen in mature human platelets (Figure S3D), suggesting that VLK is likely synthesized during megakaryocyte development and distributed to nascent platelets.

VLK phosphorylates a variety of extracellular proteins

Existing phospho-proteomic datasets pointed to a wide range of secreted proteins that are phosphorylated on tyrosine in vivo. We used these data as a guide to the testing of candidate substrates for VLK phosphorylation, with a focus on phosphorylations that had been reported at sites conserved across broad sets of related proteins. MMPs were strong candidates in this regard, due to the numerous reports of tyrosine phosphorylation in vivo of the hemopexin domain in a subset of MMPs (Figure 1A). Co-expression of VLK with hMMP1, hMMP13, hMMP14, hMMP19 and bMMP27 in each case resulted in tyrosine phosphorylation of the MMP (Figure 4A and Figure S4A). Additional substrates, structurally unrelated to MMPs, but suggested as potential substrates by reports of tyrosine phosphorylation in vivo (laminin A1, FUT8, TNFSF10) (Hornbeck et al., 2004), were also phosphorylated when co-expressed with VLK (Figure 4A and Figure S4A), suggesting that VLK substrates are not limited to a single class of proteins or structural motifs. Phosphorylation of co-expressed proteins was not seen with VLK mutated at a key residue (Lys166) in the predicted ATP binding site (VLK^{KM}) (Kinoshita et al., 2009), or with VLK^{-SP} (Figures 4A and 4B). In addition to its predicted kinase domain and signal peptide, a distinctive feature of VLK, conserved across mammalian orthologues, is a proline glycine

(PG) rich domain N-terminal to the start of the kinase domain (Figure 2A). Deletion of this conserved PG rich region ($VLK^{\Delta PG}$) also eliminates the phosphorylation of co-expressed substrates (Figure S4B), as well as of $VLK^{\Delta PG}$ itself (Figure S4C). To examine whether endogenous, as well as ectopically expressed VLK, could support tyrosine phosphorylation of a secreted protein, we tested the effect of down regulation of endogenous VLK on MMP13 phosphorylation. shRNA-mediated down-regulation of VLK reduced expression of endogenous VLK in 293T cells, with a concomitant reduction in the tyrosine phosphorylation of tagged MMP13 in the CM of transfected cells (Figure 4C).

MMP1 has been reported to be tyrosine phosphorylated in leukocytes *in vivo* on tyrosine Y360 (Raijmakers et al., 2010). Examination of material released from platelets established that endogenous MMP1 is tyrosine phosphorylated in platelets, and is released in response to stimulation by TRAP (Figure 4D). To determine whether VLK can phosphorylate MMP1 on the same site reported in vivo, we performed LC-MS/MS on a protein sample of MMP1 that had been co-expressed with VLK, and found that Y360 is the major tyrosine phosphorylated site in MMP1 under these conditions (Figure S4D) indicating that the principle site of MMP1 phosphorylation by VLK is identical to that reported in vivo. Expression of MMP1 in which Y360 was mutated to phenylalanine showed only partially reduced tyrosine phosphorylation in the presence of VLK, however, indicating that additional sites are tyrosine phosphorylated (Figure S4E), apparently below the detection limit of tandem mass spectrometry. LC-MS/MS analysis also identified phosphorylated serine (S57) and threonine (T274) residues in MMP1 (Figure S4D); raising the possibility that VLK modifies serine and threonine as well as tyrosine residues in MMP1.

VLK kinase activity is necessary for its release from the cell

Wild type VLK accumulates in the CM of cells in which it is ectopically expressed. In striking contrast, very little VLK^{KM} is secreted into CM (Figure 4E), suggesting that VLK kinase activity is necessary for its secretion. To confirm the importance of the kinase domain in VLK secretion, we generated two additional VLK mutants in which highly conserved residues in the predicted kinase domain were modified (VLK^{EA}, VLK^{DA}). Like VLK^{KM}, VLK^{EA} and VLK^{DA} failed to autophosphorylate on tyrosine, and lost its ability to phosphorylate substrates like MMP1 (Figure S4F, upper panel and S4H, left panel). Secretion of VLK^{EA} or VLK^{DA} into CM was also dramatically reduced relative to wild type VLK (Figure 4E, S4G). Examination of PK sensitivity of wild type and kinase dead VLK mutants in detergent free cell lysates demonstrated that the mutants are localized to the ER/Golgi lumen as efficiently as wild type VLK (Figure 4E). VLK has been previously reported to be phosphorylated at Y148 (Kinoshita et al., 2009); mutation of this site to phenylalanine does not alter VLK secretion, indicating that autophosphorylation at this site is not involved in the control of VLK progression through the secretory pathway (Figure 4E). Mutation of Y148 also had no effect on the ability of VLK to phosphorylate co-expressed substrates (Figure S4I, upper panel). Tyrosine phosphorylation of VLK was still seen in the Y148F mutant, but not in the kinase dead VLK constructs, suggesting the existence of additional VLK auto-phosphorylation sites (Figures S4F and I, lower panels). These data indicate that VLK kinase activity is important for its release from the cell, but whether this is due to

autophosphorylation on uncharacterized sites or to phosphorylation of other substrates in the secretory pathway is not clear.

VLK phosphorylates endogenous proteins in the secretory pathway

To define the spectrum of VLK-dependent phosphorylation of endogenous proteins, we generated K4 fibroblasts stably expressing either VLK or VLK^{KM}. Expression of wild type VLK, but not VLK^{KM}, resulted in a dramatic increase in total cellular tyrosine phosphoprotein (Figure 5A). Because VLK diverges strongly from cytosolic tyrosine protein kinases in its kinase domain, we expected that VLK kinase activity would not be reduced by inhibitors of cytosolic tyrosine kinases. Treatment of cells with the broad spectrum tyrosine kinase inhibitor dasatinib (Shah et al., 2004) strongly reduced tyrosine phosphoprotein bands shared among GFP K4, VLK K4 and VLK^{KM} K4 cells, but had no effect on bands unique to VLK K4 cells (Figure 5A). Tyrosine phosphorylation in the CM of K4 cells was exclusively detected following VLK but not VLK^{KM} overexpression (Figure 5B), and the accumulation of these proteins in CM was abrogated by treatment with brefeldin A (Figure S5C). VLK-dependent phosphorylations were, like VLK itself, protected from PK treatment in detergent free lysates (Figure S5A). No changes in threonine or serine phosphorylations in K4 cell lysates were seen following VLK overexpression (Figure S5B) and no serine or threonine phosphorylations at all could be detected in the CM of K4 cells (Figure S5C). The predominant detectable change in overall protein phosphorylation associated with VLK expression therefore appears to be phosphotyrosine.

To identify endogenous substrates for VLK, we did anti-phosphotyrosine (anti-pTyr) immunoprecipitation of tryptic digests of total cell protein and total CM from VLK expressing cells, followed by identification of phosphopeptides by LC-MS/MS (Breitkopf and Asara, 2012). 140 distinct tyrosine phosphopeptides were identified, of which 48 tyrosine phosphopeptides mapped either to proteins containing canonical signal peptides or to the extracellular domains of transmembrane proteins (Table 1 and Supplementary File 1). In addition, several tyrosine phosphopeptides were exclusively detected in CM (Table 1). Of the 48 secretory/extracellular tyrosine phosphopeptides identified, 11 have been identified in biosamples from in vivo tissue (Hornbeck et al., 2004). The most frequently detected phosphopeptide in VLK expressing K4 cells occurred at Tyr66 in ERP29, an ER chaperone that is the mammalian homologue of the Drosophila developmental mutation *windbeutel*. Tyr66, conserved in *windbeutel*, is essential for chaperone function (Barnewitz et al., 2004), and has been found to be phosphorylated both in vivo and in a wide range of cell lines in 366 separate MS-MS analyses (Bai et al., 2012; Ding et al., 2011; Gu et al., 2011; Guo et al., 2008; Hornbeck et al., 2004; Rikova et al., 2007). These observations indicate that the tyrosine phosphorylation of specific sites in secretory pathway proteins occurs in vivo under endogenous conditions of VLK expression as well as in the presence of ectopic VLK. Comparison of tyrosine phosphorylation in cells expressing wild type VLK or VLK^{KM} using SILAC analysis established that only proteins in the secretory pathway, and not cytoplasmic proteins, showed increased tyrosine phosphorylation in the presence of wild type VLK, confirming that VLK is likely to act specifically on proteins in the secretory pathway (Table S1). To further confirm that the tyrosine phosphorylation in secretory pathway proteins was VLK dependent, we performed immunoblot analysis for several

candidate VLK substrates on phospho-tyrosine immunoprecipitation of lysates from K4 VLK or K4 VLK^{KM} cells (Figure S5D). In each case, tyrosine phosphorylation was seen only in material from cells expressing active VLK. Systematic comparison of tyrosine phosphopeptides identified in cells expressing wild type VLK and cells expressing VLK^{KM} established that every peptide annotated as deriving from a secreted protein or from the extracellular domain of a transmembrane protein was detected only in cells expressing wild type VLK (data not shown).

To confirm that tyrosine kinase activity is intrinsic to VLK, and is inactivated by mutation of Lys166, wild type and mutant proteins were expressed and purified in a baculoviral expression system. Wild type, but not mutant, VLK protein, phosphorylated both a peptide derived from the hemopexin domain of MMP1 (HpxPeptide) or a peptide designed as an optimal substrate based on tyrosine phosphopeptides identified in VLK expressing cells(Ypeptide) (Figure 6A). Wild type, but not mutant, VLK protein also directly phosphorylated purified ERP29 protein, and showed dramatically reduced phosphorylation of mutant ERP29 lacking the sites of tyrosine phosphorylation identified in VLK expressing cells and in vivo (Tyr64/Tyr66) (Figure 6B). Kinase activity was optimal in the presence of a combination of Mg^{2+} and Ca^{2+}, and was only weakly supported by Mn^{2+} (Figure S6). The specific activity of VLK purified from the baculoviral system, ~0.2 nmol/min/mg, was 10–100 fold lower than activities typically observed for cytoplasmic tyrosine kinases. VLK phosphorylated peptides in which the tyrosine residue was replaced with serine (Speptide) or threonine (Tpeptide) with reduced efficiency (Figure 6A). These data establish that purified VLK has intrinsic kinase activity with a preference for tyrosine, and can phosphorylate ERP29 on the same tyrosines shown to be phosphorylated in VLK-expressing cells and in vivo.

DISCUSSION

There is a broad range of potential substrates for VLK in vitro and in vivo

Examination of an unbiased set of secreted and lumenal tyrosine phosphoproteins from VLK expressing cells reveals a broad set of VLK substrates, many of which correspond to tyrosine phosphorylations in secreted proteins seen in vivo (Table I). While our initial studies focused on the MMPs as candidate substrates, the broader set of substrates we find in VLK-expressing fibroblasts do not share evident structural features with MMP hemopexin domains or with one another. They also do not share any clear local motif surrounding the phosphorylation site, in contrast to the secreted serine kinase FAM20C, which targets the local motif SerXGlu (Tagliabracci et al., 2012). The basis for substrate recognition by the VLK kinase activity therefore remains puzzling. Phosphorylation by VLK may depend on some combination of surface exposure of tyrosine, juxtaposition of specific amino acids in the tertiary structure around the phosphorylation site, and/or association of VLK with multiple targeting subunits that direct the kinase activity to distinct classes of substrates.

Ectopic expression of VLK in fibroblasts substantially alters total cellular protein tyrosine phosphorylation (Figure 5A), establishing that VLK dependent phosphorylation in cells is quantitatively similar to the sum of activities of endogenous cytosolic tyrosine kinases. The low activity of purified VLK protein therefore seems paradoxical, and may reflect inefficient

folding or disulfide bond formation in the ER in the baculovirus/S9 system, lack of a lumenal modifying enzyme required for optimal activity in this system, or lack of an important mammalian modifying subunit or co-factor. Elimination of the VLK signal peptide eliminates VLK autophosphorylation (Figures 2 and 4), suggesting that lumenal chaperones or other lumenal proteins are essential for proper folding/activity of the secreted kinase domain. Phosphorylation by VLK may also require secondary or tertiary structural features of the substrate that are not recapitulated by the test substrates used here. A third possibility is that lumenal tyrosine phosphatase activity may be much lower than cytosolic tyrosine phosphatase activity, allowing a low turnover lumenal tyrosine kinase to sustain significant lumenal/secreted tyrosine phosphorylation. Defining the mechanism by which VLK activity and substrate recognition are regulated will be an important area for further investigation.

Relationship between VLK substrates and phenotype

Loss of VLK results perinatal lethality, with defects in skeletal, lung, and craniofacial development (Imuta et al., 2009; Kinoshita et al., 2009; Melvin et al., 2013; Probst et al., 2013). During embryogenesis, VLK is expressed strongly in mesenchymal condensations in bone, lung, and elsewhere, and is broadly expressed at lower levels in the adult (Kinoshita et al., 2009) (Imuta et al., 2009). In human genome wide association studies (GWAS), VLK has been correlated with variations in bone density, consistent with a role in skeletal development or homeostasis (Hsu and Kiel, 2012). At the cellular level, the best characterized defect in VLK null animals is in the differentiation of chondrocytes in long bones (Imuta et al., 2009; Kinoshita et al., 2009; Probst et al., 2013). Our studies identify a variety of VLK substrates with established roles in skeletal development, including Type I collagen (Prockop, 1985), MMP13 (Stickens et al., 2004), MIA3 (an essential collagen chaperone) (Wilson et al., 2011), and MESD (a chaperone for the wnt receptor LRP5/6) (Hsieh et al., 2003; Zhang et al., 2004). At this point, it is not possible to assess whether the VLK null phenotype reflects a major functional alteration in one or two key substrates, or more subtle changes in a broad set of functionally interacting substrates that collectively impact chondrocyte differentiation.

Our work also suggests a role for VLK in platelet function. VLK protein is present in greater relative abundance in platelets than in any other cell type we have examined, and is quantitatively released following stimulation of platelet degranulation. Proteins secreted following platelet degranulation regulate thrombosis, angiogenesis, inflammation, and tissue remodeling during wound healing and tumorigenesis (Blair and Flaumenhaft, 2009; Harrison and Cramer, 1993). MMP1, which we have found to be directly phosphorylated on tyrosine by VLK, is also co-secreted from platelets with VLK, and is tyrosine phosphorylated in this releasate (Figure 4D) as well as in vivo (Raijmakers et al., 2010). MMP1 has a role both in platelet activation itself (Santos-Martinez et al., 2008) and in tissue remodeling associated with wound healing (Raffetto and Khalil, 2008). In addition, a variety of secreted proteins involved in the regulation of thrombosis are tyrosine phosphorylated in vivo (Hornbeck et al., 2004), suggesting a potential role for secreted tyrosine kinase activity in the control of thrombosis.

Potential Functions of VLK Phosphorylation sites

The number and diversity of VLK phosphorylation sites that we have identified makes it difficult to generalize regarding the potential function of these phosphorylations. In a number of instances, however, existing structural or functional information points to specific roles for some of the tyrosine phosphorylations we have found. The tyrosine phosphorylation in osteopontin, for example, occurs in the middle of the integrin alpha9beta1 binding sequence SVVY*GLR, and the addition of a charged phosphate in the middle of this short recognition site would be strongly predicted to modulate integrin binding (Yokasaki and Sheppard, 2000). The phosphorylation site in Type I collagen occurs 2 amino acids N-terminal to the C-propeptide maturation site (Kessler et al., 1996), pointing to a role for tyrosine phosphorylation in collagen maturation.

Resident ER chaperones comprise a significant portion of VLK substrates, suggesting that at least one function of VLK is to modulate the secretory pathway. In the cases of the ER chaperones MESD and ERP29, the tyrosines that we have found to be phosphorylated are each established to have an essential role in the interaction of these chaperones with specific substrates (Barak et al., 2009; Chen et al., 2010; Chen et al., 2011), also indicating a role for phosphorylation in the modulation of core protein function. Kinoshita et al. previously reported that ectopic VLK expression reduced the overall rate of VSVG progression through the secretory pathway (Kinoshita et al., 2009), but this effect was seen equally with expression of wt VLK and VLK^{KM}, the latter of which we have found to be inactive as a protein kinase. This general effect on secretion therefore appears not to be associated with the catalytic activity of VLK, but whether VLK selectively modifies chaperone function by phosphorylation remains an open question. Ectopically expressed VLK has been reported to co-localize with Golgi markers in immunohistochemical analysis (Kinoshita et al., 2009). The abundant phosphorylation of ER resident proteins we find in VLK expressing cells indicates that VLK is either present and active in the ER or that it phosphorylates these proteins in the Golgi during cycling between these compartments. Interestingly, we find that VLK mutants that lack kinase activity are no longer efficiently secreted from the cell, suggesting that the catalytic activity of VLK modulates its own progression through the secretory pathway.

Does VLK function in the secretory pathway, or outside the cell, or both?

The characterization of FAM20C as an active serine kinase in the Golgi apparatus provides a clear precedent that ATP dependent protein phosphorylation can take place in the secretory apparatus (Tagliabracci et al., 2012; Yalak and Vogel, 2012). Under the conditions of our experiments in fibroblasts, there is no significant source of ATP outside the cell, and therefore the VLK phosphorylations we observe in these cells are likely to take place within the secretory pathway. In platelets, however, dense granules provide an abundant local source of extracellular ATP following stimulation of secretion (McNicol and Israels, 1999). We find that this endogenous ATP pool can sustain de novo tyrosine phosphorylation of secreted proteins in platelet releasate (Figure 3B) indicating that secreted tyrosine kinase activity can be supported by an endogenous source of extracellular ATP. The concentration of ATP in platelet dense granules is estimated to be 0.1–1M (Gordon, 1986; McNicol and Israels, 1999), and the local concentration of ATP following degranulation in thromboses

has been estimated to be well within the range required for protein kinase activity (Gordon, 1986). The co-release of VLK and ATP from platelets therefore provides the first plausible example of de novo tyrosine phosphorylation in the extracellular environment. ATP release from a broad range of both excitable and non-excitable cell types is also well known, and has been proposed to support extracellular kinase activity (Praetorius and Leipziger, 2009; Yalak and Vogel, 2012). Extracellular ATP has also been reported to be elevated in the tumor microenvironment (Pellegatti et al., 2008), and to have a variety of pro-tumorigenic properties (Braganhol et al., 2012; Stagg and Smyth, 2010). Tyrosine phosphorylation on ER-resident proteins such as ERP29 in vitro and in vivo indicates that endogenous VLK has significant activity intracellularly in the secretory pathway, but the regulated secretion of active VLK from platelets points strongly to a significant role for VLK in the extracellular environment as well.

Patterns of Secreted Tyrosine phosphorylation in vivo

A striking feature of tyrosine phosphorylation of secreted proteins in vivo is the conservation of phosphorylation sites in specific structural motifs in distantly related proteins. In addition to the hemopexin domains noted in the Introduction, immunoglobulin (IG) domains, fibronectin (FN3) domains, and VWA domains, are phosphorylated on tyrosine in multiple proteins, and these phosphorylations can be localized to conserved structural features within the domain (Figure S1). Conserved tyrosine phosphorylation of modular domains in ECM proteins (Hynes and Naba, 2011) in vivo can be found in many additional domain classes (e.g. CUB, TSP1, FN1, FN2), but in these cases insufficient structural information is available to place the phosphorylations relative to a common structural feature. While VLK may be responsible for many of these phosphorylations, additional secreted kinases may be responsible for the full spectrum of secreted tyrosine phosphorylations reported in vivo. Bioinformatic work has identified several other putative secreted proteins with significant similarity to VLK, suggesting that the secreted protein kinome may still be far from complete (Dudkiewicz et al., 2013; Tagliabracci et al., 2013).

Conclusion

Our work places VLK with FAM20C as members of a new class of regulator of extracellular proteins, the secreted protein kinases. Fam20C and VLK are evolutionarily more distant from one another than they are from canonical cytoplasmic kinases, suggesting that the evolution of multiple secreted kinases occurred as independent events rather than divergence from a primordial secreted kinase. The breadth and conservation of extracellular structural and regulatory proteins that are found to be phosphorylated in vivo makes it likely that defining the physiological roles of secreted protein kinases will have a major impact on our understanding of both normal tissue homeostasis and disease pathogenesis.

Experimental procedures

Proteinase K protection assay and PNGase F treatment

Cells were incubated in an isotonic extraction buffer and lysed using a Dounce homogenizer, incubated in the presence of proteinase K alone or in combination with 1% NP40. Proteolysis was terminated with PMSF and samples analyzed by Western blot.

Analysis of ectopically expressed VLK with candidate substrates

293T cells were transfected with cDNAs encoding wild type or mutant VLK constructs with or without co-transfection of candidate epitope tagged substrates. After 24–48 hrs cell lysates or CM were immunoprecipitated with anti-tag antibody and analyzed for total expression or tyrosine phosphorylation by Western blot.

Microscopy

Platelets were fixed in 4% formaldehyde and centrifuged onto poly-L-lysine (1 μg/mL)-coated coverslides, and analyzed for immunofluorescence with anti-VLK antibody by scanning confocal microscopy as described in Extended Experimental Procedures. Rapid-freeze immunogold electron microscopy of washed human platelets was performed as previously described (Italiano et al., 1999).

Platelet release assay

Whole blood was collected and platelets were isolated as previously described (Thon et al., 2012). Platelets were treated with 0.2 μM phorbol-12-myristate-13-acetate (PMA) or 0.5 μM thrombin-receptor activating peptide (TRAP) to induce degranulation. After 4′ of treatment, cells were removed by centrifugation, and the supernatant (releasate) was treated with ATP, apyrase, or anti-VLK antibody as described in legend to Figure 3.

Kinase assays

Mus musculus VLK (amino acids 55–492) was cloned into a modified pl-secSUMOstar vector (LifeSensors) containing a tobacco etch virus (TEV) cleavage site, and recombinant protein purified from CM as described in extended experimental procedures. For peptide kinase assays, 0.1 μg of purified recombinant protein was incubated with 5 μM ^{33}[P]-γ-ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 50 mM Tris pH 7.5, and 1 mM biotinylated peptide, and then biotinylated peptide was collected on streptavidin agarose and counted in a liquid scintillation counter. For ERP29 phosphorylation assays, purified wild type or mutant ERP29 was assayed as substrate for phosphorylation by purified VLK as described above, and analyzed by Laemmli gel electrophoresis and autoradiography.

Mass spectrometry

K4 fibroblasts stably expressing retrovirally transduced wild type VLK or VLK^{KM} were lysed with urea lysis buffer, digested with trypsin, and tyrosine phosphopeptides isolated by immunoprecipitation and analyzed as described in extended experimental procedures. CM from transduced fibroblasts was precipitated with TCA and then solubilized and analyzed as for cell lysates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Min Yuan for help with mass spectrometry experiments, Peter Hornbeck (Cell Signaling Technologies, Inc.) for valuable discussions, Ross Okazaki and Rajesh Kulenthirarajan for platelet isolation, and Shin-Ichi Nishikawa (RIKEN) for cDNAs encoding wild type human VLK and VLK^{KM}. This work was supported in part by the National Institutes of Health grants GM089885 (M.W.), Hl68130 (J.E.I.), 1K99HL114719-01A1 (J.N.T), 5P30CA006516, 5P01CA120964, NIH shared instrumentation grant 1S10OD010612 (J.M.A.), DK 18024 and DK 18849 (J.E.D.), and grants from the Korean Government to C.Y.Y. (NRF 2012R1A5A1048236 and 2012R1A2A2A01046485, Next Generation BioGreen 21 Program PJ00812701). M.R.B. was supported by a Swiss National Science Foundation fellowship.

References

Ahn HS, Foster C, Boykow G, Arik L, Smith-Torhan A, Hesk D, Chatterjee M. Binding of a thrombin receptor tethered ligand analogue to human platelet thrombin receptor. Mol Pharmacol. 1997; 51:350–356. [PubMed: 9203642]

Bai Y, Li J, Fang B, Edwards A, Zhang G, Bui M, Eschrich S, Altiok S, Koomen J, Haura EB. Phosphoproteomics identifies driver tyrosine kinases in sarcoma cell lines and tumors. Cancer Res. 2012; 72:2501–2511. [PubMed: 22461510]

Ballif BA, Carey GR, Sunyaev SR, Gygi SP. Large-scale identification and evolution indexing of tyrosine phosphorylation sites from murine brain. J Proteome Res. 2008; 7:311–318. [PubMed: 18034455]

Barak NN, Neumann P, Sevvana M, Schutkowski M, Naumann K, Malesevic M, Reichardt H, Fischer G, Stubbs MT, Ferrari DM. Crystal structure and functional analysis of the protein disulfide isomerase-related protein ERp29. J Mol Biol. 2009; 385:1630–1642. [PubMed: 19084538]

Barnewitz K, Guo C, Sevvana M, Ma Q, Sheldrick GM, Soling HD, Ferrari DM. Mapping of a substrate binding site in the protein disulfide isomerase-related chaperone wind based on protein function and crystal structure. J Biol Chem. 2004; 279:39829–39837. [PubMed: 15252019]

Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 2009; 23:177–189. [PubMed: 19450911]

Braganhol E, Wink MR, Lenz G, Battastini AM. Purinergic signaling in glioma progression. Adv Exp Med Biol. 2012; 986:81–102. [PubMed: 22879065]

Breitkopf SB, Asara JM. Determining in vivo phosphorylation sites using mass spectrometry. Curr Protoc Mol Biol. 2012; Chapter 18(Unit 18 19):11–27.

Brown J, Novak EK, Takeuchi K, Moore K, Medda S, Swank RT. Lumenal location of the microsomal beta-glucuronidase-egasyn complex. J Cell Biol. 1987; 105:1571–1578. [PubMed: 3667691]

Chen J, Li Q, Liu CC, Zhou P, Bu G, Wang J. NMR structure note: solution structure of the core domain of MESD that is essential for proper folding of LRP5/6. J Biomol NMR. 2010; 47:283–288. [PubMed: 20506034]

Chen J, Liu CC, Li Q, Nowak C, Bu G, Wang J. Two structural and functional domains of MESD required for proper folding and trafficking of LRP5/6. Structure. 2011; 19:313–323. [PubMed: 21397183]

Ding VM, Boersema PJ, Foong LY, Preisinger C, Koh G, Natarajan S, Lee DY, Boekhorst J, Snel B, Lemeer S, et al. Tyrosine phosphorylation profiling in FGF-2 stimulated human embryonic stem cells. PLoS One. 2011; 6:e17538. [PubMed: 21437283]

Dudkiewicz M, Lenart A, Pawlowski K. A novel predicted calcium-regulated kinase family implicated in neurological disorders. PLoS One. 2013; 8:e66427. [PubMed: 23840464]

Faber HR, Groom CR, Baker HM, Morgan WT, Smith A, Baker EN. 1.8 A crystal structure of the C-terminal domain of rabbit serum haemopexin. Structure. 1995; 3:551–559. [PubMed: 8590016]

Fantl W, Johnson D, Williams L. Signalling By Receptor Tyrosine Kinases. Annu Rev Biochem. 1993; 62:453–481. [PubMed: 7688944]

Fujisawa R, Tamura M. Acidic bone matrix proteins and their roles in calcification. Front Biosci (Landmark Ed). 2011; 17:1891–1903. [PubMed: 22201843]

Gomis-Ruth FX, Gohlke U, Betz M, Knauper V, Murphy G, Lopez-Otin C, Bode W. The helping hand of collagenase-3 (MMP-13): 2.7 A crystal structure of its C-terminal haemopexin-like domain. J Mol Biol. 1996; 264:556–566. [PubMed: 8969305]

Goncalves L, Filipe M, Marques S, Salgueiro AM, Becker JD, Belo JA. Identification and functional analysis of novel genes expressed in the Anterior Visceral Endoderm. Int J Dev Biol. 2011; 55:281–295. [PubMed: 21553379]

Gordon JL. Extracellular ATP: effects, sources and fate. Biochem J. 1986; 233:309–319. [PubMed: 3006665]

Gu TL, Deng X, Huang F, Tucker M, Crosby K, Rimkunas V, Wang Y, Deng G, Zhu L, Tan Z, et al. Survey of tyrosine kinase signaling reveals ROS kinase fusions in human cholangiocarcinoma. PLoS One. 2011; 6:e15640. [PubMed: 21253578]

Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, et al. Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci U S A. 2008; 105:692–697. [PubMed: 18180459]

Hanks SK. Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. Genome Biol. 2003; 4:111. [PubMed: 12734000]

Harrison P, Cramer EM. Platelet alpha-granules. Blood Rev. 1993; 7:52–62. [PubMed: 8467233]

Hornbeck PV, Chabra I, Kornhauser JM, Skrzypek E, Zhang B. PhosphoSite: A bioinformatics resource dedicated to physiological protein phosphorylation. Proteomics. 2004; 4:1551–1561. [PubMed: 15174125]

Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K. WoLF PSORT: protein localization predictor. Nucleic acids research. 2007; 35:W585–587. [PubMed: 17517783]

Hsieh JC, Lee L, Zhang L, Wefer S, Brown K, DeRossi C, Wines ME, Rosenquist T, Holdener BC. Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell. 2003; 112:355–367. [PubMed: 12581525]

Hsu YH, Kiel DP. Genome-Wide Association Studies of Skeletal Phenotypes: What We Have Learned and Where We Are Headed. J Clin Endocrinol Metab. 2012

Hynes RO, Naba A. Overview of the matrisome--an inventory of extracellular matrix constituents and functions. Cold Spring Harb Perspect Biol. 2011; 4:a004903. [PubMed: 21937732]

Imuta Y, Nishioka N, Kiyonari H, Sasaki H. Short limbs, cleft palate, and delayed formation of flat proliferative chondrocytes in mice with targeted disruption of a putative protein kinase gene, Pkdcc (AW548124). Dev Dyn. 2009; 238:210–222. [PubMed: 19097194]

Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. J Cell Biol. 1999; 147:1299–1312. [PubMed: 10601342]

Jozic D, Bourenkov G, Lim NH, Visse R, Nagase H, Bode W, Maskos K. X-ray structure of human proMMP-1: new insights into procollagenase activation and collagen binding. J Biol Chem. 2005; 280:9578–9585. [PubMed: 15611040]

Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS. Bone morphogenetic protein-1: the type I procollagen C-proteinase. Science. 1996; 271:360–362. [PubMed: 8553073]

Kinoshita M, Era T, Jakt LM, Nishikawa S. The novel protein kinase Vlk is essential for stromal function of mesenchymal cells. Development. 2009; 136:2069–2079. [PubMed: 19465597]

Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol. 2011:3.

McNicol A, Israels SJ. Platelet dense granules: structure, function and implications for haemostasis. Thromb Res. 1999; 95:1–18. [PubMed: 10403682]

Melvin VS, Feng W, Hernandez-Lagunas L, Artinger KB, Williams T. A morpholino-based screen to identify novel genes involved in craniofacial morphogenesis. Dev Dyn. 2013; 242:817–831. [PubMed: 23559552]

Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS One. 2008; 3:e2599. [PubMed: 18612415]

Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011; 8:785–786. [PubMed: 21959131]

Praetorius HA, Leipziger J. ATP release from non-excitable cells. Purinergic Signal. 2009; 5:433–446. [PubMed: 19301146]

Probst S, Zeller R, Zuniga A. The hedgehog target Vlk genetically interacts with Gli3 to regulate chondrocyte differentiation during mouse long bone development. Differentiation. 2013; 85:121–130. [PubMed: 23792766]

Prockop DJ. Mutations in collagen genes. Consequences for rare and common diseases. J Clin Invest. 1985; 75:783–787. [PubMed: 3884665]

Raffetto JD, Khalil RA. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. Biochem Pharmacol. 2008; 75:346–359. [PubMed: 17678629]

Raijmakers R, Kraiczek K, de Jong AP, Mohammed S, Heck AJ. Exploring the human leukocyte phosphoproteome using a microfluidic reversed-phase-TiO2-reversed-phase high-performance liquid chromatography phosphochip coupled to a quadrupole time-of-flight mass spectrometer. Anal Chem. 2010; 82:824–832. [PubMed: 20058876]

Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007; 131:1190–1203. [PubMed: 18083107]

Rinschen MM, Yu MJ, Wang G, Boja ES, Hoffert JD, Pisitkun T, Knepper MA. Quantitative phosphoproteomic analysis reveals vasopressin V2-receptor-dependent signaling pathways in renal collecting duct cells. Proc Natl Acad Sci U S A. 2010; 107:3882–3887. [PubMed: 20139300]

Rozenvayn N, Flaumenhaft R. Protein kinase C mediates translocation of type II phosphatidylinositol 5-phosphate 4-kinase required for platelet alpha-granule secretion. J Biol Chem. 2003; 278:8126–8134. [PubMed: 12509423]

Santos-Martinez MJ, Medina C, Jurasz P, Radomski MW. Role of metalloproteinases in platelet function. Thromb Res. 2008; 121:535–542. [PubMed: 17681591]

Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science. 2004; 305:399–401. [PubMed: 15256671]

Stagg J, Smyth MJ. Extracellular adenosine triphosphate and adenosine in cancer. Oncogene. 2010; 29:5346–5358. [PubMed: 20661219]

Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Development. 2004; 131:5883–5895. [PubMed: 15539485]

Tagliabracci VS, Engel JL, Wen J, Wiley SE, Worby CA, Kinch LN, Xiao J, Grishin NV, Dixon JE. Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. Science. 2012; 336:1150–1153. [PubMed: 22582013]

Tagliabracci VS, Pinna LA, Dixon JE. Secreted protein kinases. Trends Biochem Sci. 2013; 38:121–130. [PubMed: 23276407]

Thon JN, Macleod H, Begonja AJ, Zhu J, Lee KC, Mogilner A, Hartwig JH, Italiano JE Jr. Microtubule and cortical forces determine platelet size during vascular platelet production. Nat Commun. 2012; 3:852. [PubMed: 22617292]

Wilson DG, Phamluong K, Li L, Sun M, Cao TC, Liu PS, Modrusan Z, Sandoval WN, Rangell L, Carano RA, et al. Global defects in collagen secretion in a Mia3/TANGO1 knockout mouse. J Cell Biol. 2011; 193:935–951. [PubMed: 21606205]

Yalak G, Vogel V. Extracellular phosphorylation and phosphorylated proteins: not just curiosities but physiologically important. Sci Signal. 2012; 5:re7. [PubMed: 23250399]

Yokasaki Y, Sheppard D. Mapping of the cryptic integrin-binding site in osteopontin suggests a new mechanism by which thrombin can regulate inflammation and tissue repair. Trends Cardiovasc Med. 2000; 10:155–159. [PubMed: 11239795]

Zhang Y, Wang Y, Li X, Zhang J, Mao J, Li Z, Zheng J, Li L, Harris S, Wu D. The LRP5 high-bone-mass G171V mutation disrupts LRP5 interaction with Mesd. Mol Cell Biol. 2004; 24:4677–4684. [PubMed: 15143163]

Highlights

- VLK is a secreted tyrosine kinase
- VLK phosphorylates proteins in the secretory pathway and outside the cell.
- VLK is released from platelets alpha-granules in response to physiological stimuli
- ATP co-released with VLK during secretion supports extracellular phosphorylation.

A

MMP1	(Tyr360)	GQNVLHG**Y***PKDIYSS
MMP1B	(Tyr360)	TRSMDPG**Y***PRLIAED
MMP12	(Tyr414)	RQMMDPG**Y***PKLITKN
MMP13	(Tyr366)	GYDILEG**Y***PKKISEL
MMP16	(Tyr521)	ILKVEPG**Y***PRSILKD
MMP16	(Tyr377)	NNRVMDG**Y***PMQITYF
MMP24	(Tyr534)	AFISKEG**Y***YTYFYKG
MMP27	(Tyr360)	GYAVLPD**Y***PKSIHTL
MMP14	(Tyr353)	NNQVMDG**Y***PMPIGQF
Hemopexin	(Tyr341)	GYTLVSG**Y***PKRLEKE
Lubricin	(Tyr1228)	NDVMDAG**Y***PKLIVKG

B

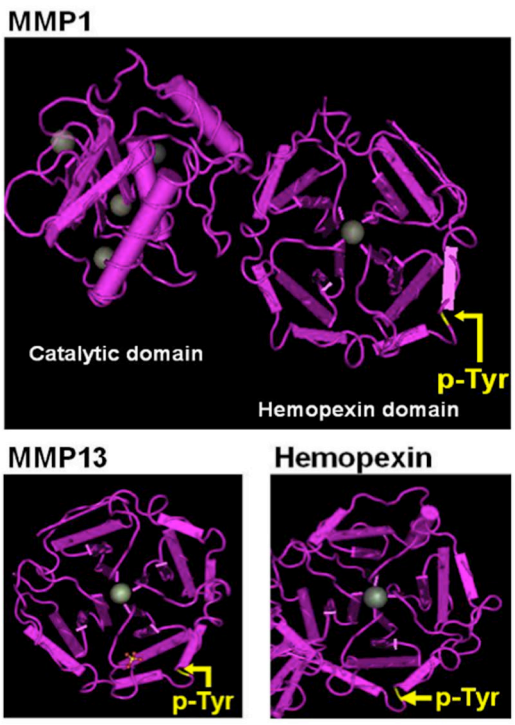

Figure 1. Conserved position of phosphorylated tyrosine in hemopexin domains reported in vivo in Phosphosite compendium

A) All sites shown are at orthologous positions within the hemopexin domain as defined by primary sequence conservation. B) Hemopexin domain structures for MMP1 MMDB ID: 30864 (Jozic et al., 2005) and MMP13 (MMDB ID: 57090)(Gomis-Ruth et al., 1996) and hemopexin (MMDB ID:56395) (Faber et al., 1995). Yellow arrow indicates reported site of tyrosine phosphorylation (Hornbeck et al., 2004; Raijmakers et al., 2010). See also Figure S1.

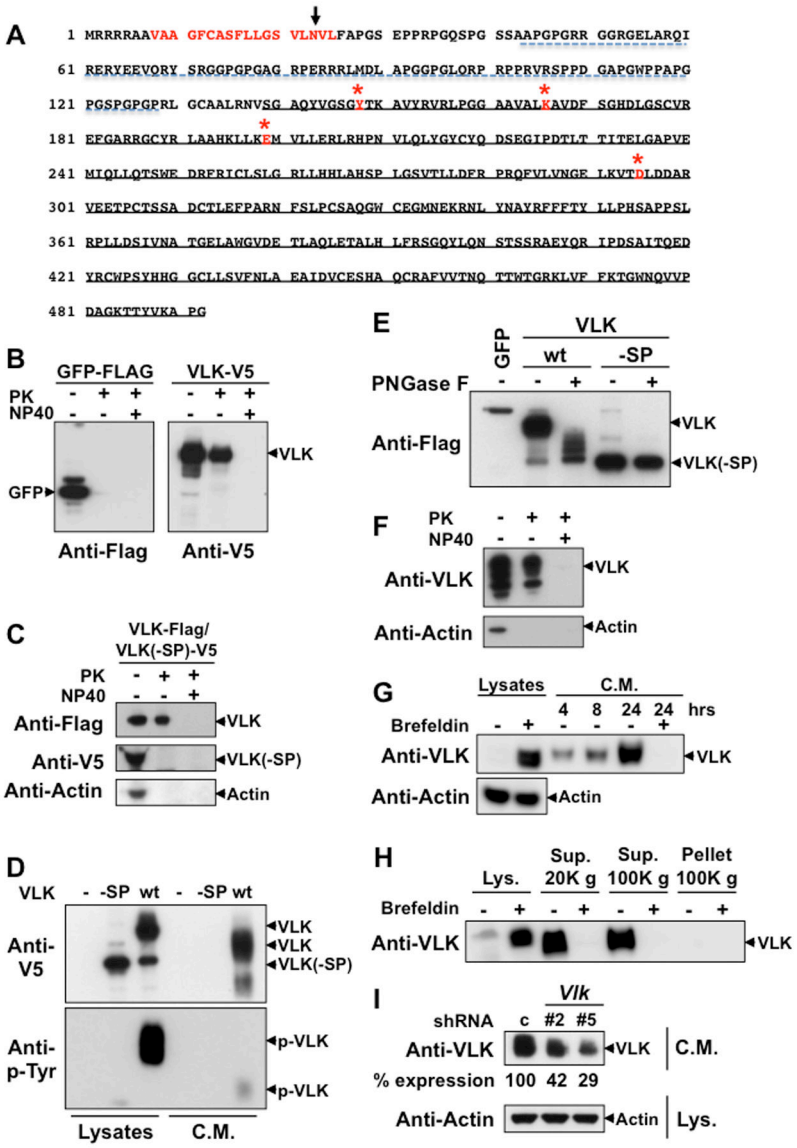

Figure 2. VLK is constitutively secreted and its glycosylation and secretory pathway localization are signal peptide dependent

A) Sequence of mouse VLK. Signal peptide is indicated in red, signal peptide cleavage site with an arrow. Sites of point mutations discussed in the text are marked in red with asterisk, the ProGly rich domain is underlined in dashed blue. The predicted kinase region is underlined in black. B) Detergent free lysates of 293T cells overexpressing GFP or VLK were treated with proteinase K (PK) alone or in combination with NP40; C) Detergent free lysates of HepG2 cells co-expressing VLK and VLK^{-SP} were treated with proteinase K (PK) alone or in combination with NP40. D) VLK expression was determined in lysates and CM of 293T cells overexpressing VLK or VLK^{-SP}. Tyrosine phosphorylation was detected following VLK immunoprecipitation. E) Cell lysates expressing either VLK or VLK^{-SP} were treated with PNGase to remove N-linked glycosylations. F) Lysates of HepG2 cells were treated with PK alone or in combination with NP40, and endogenous VLK detected by Western blot. Cytoplasmic actin was measured as a marker of cytoplasmic protein. G) VLK

expression and secretion was detected in lysates and CM of brefeldin A treated HepG2 cells. H) VLK expression was detected in lysates (Lys) and CM of HepG2 cells treated with brefeldin A, before and after ultracentrifugation. I) VLK protein levels were determined in CM of HepG2 cells stably transduced with either a scrambled shRNA (c) or two independent shRNAs targeting *Vlk* (#2, #5). Percentage of protein expression was quantified using ImageJ. See also Figure S2.

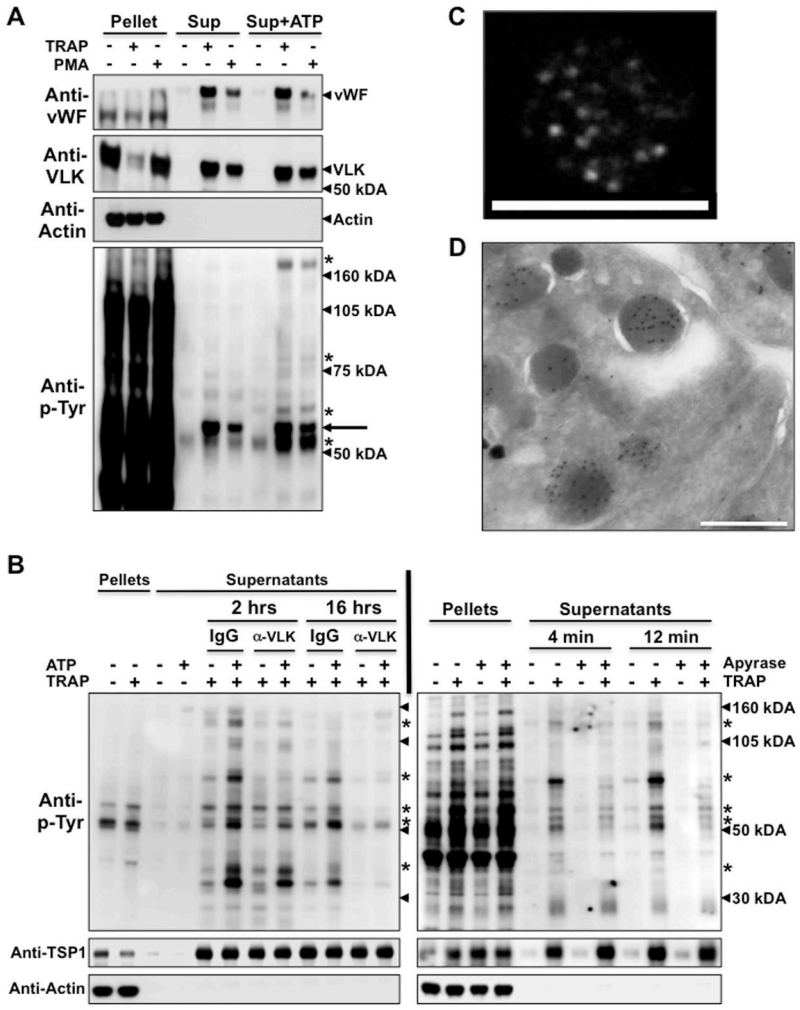

Figure 3. VLK is secreted from platelets and is responsible for tyrosine phosphorylation in the platelet releasate

A) Freshly isolated human platelets were treated with either 0.2 μM PMA or 0.5 μM TRAP for 15′. After centrifugation to remove cells, cell free releasate was incubated for an additional 15′ in the presence or absence of 2 mM ATP (Sup.+ATP). Black arrow indicates phospho-tyrosine band co-migrating with VLK. Asterisks mark bands that change in releasate with stimulation and ATP. B) Tyrosine phosphorylation is dependent on VLK and on endogenous ATP. Left Panel: Following platelet stimulation with TRAP for 4′, VLK was depleted from releasates by incubation with anti-VLK or control IgG and protein A sepharose for the indicated times, and then phosphorylation was stimulated by exogenous 2 mM ATP addition (+ATP) for 15′. Note that some VLK dependent phosphorylation occurs in the absence of exogenous ATP, presumably due to endogenous secreted ATP. Right panel: platelets were stimulated with TRAP in the presence of 100 Units of apyrase to degrade endogenous released ATP. After 4′ stimulation with TRAP, platelets were cleared by centrifugation and releasate incubated for additional time indicated, then analyzed by anti-pTyr Western blot. Asterisks indicate bands changing following treatments. C) Immunofluorescence of platelets with anti-VLK antibody shows puncate staining. D)

Immunogold-EM staining with anti-VLK localizes VLK to alpha granules. Scale bar is 5 μm in C and 0.5 μm in D. See also Figure S3.

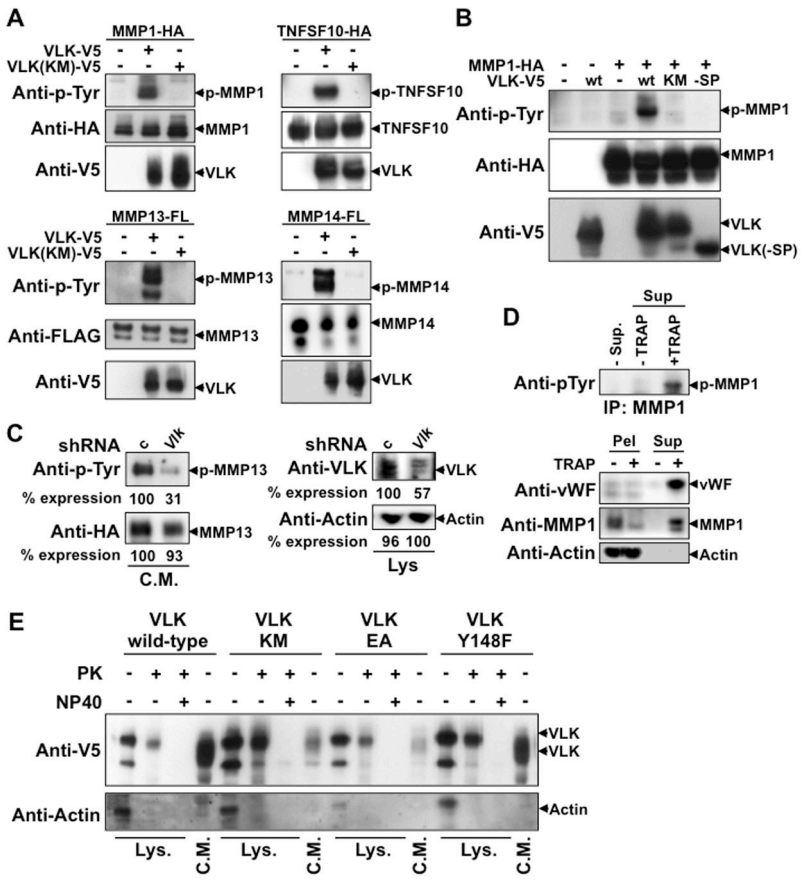

Figure 4. VLK phosphorylates secreted and transmembrane substrates

A) 293T cells were co-transfected with the indicated candidate substrate and either VLK wild-type or VLK^{KM}. Tyrosine phosphorylation was detected following immunoprecipitation of candidate substrates from cell lysates. B) Tyrosine phosphorylation was analyzed in lysates of 293T cells co-expressing MMP1 and VLK, VLK^{KM}, or VLK^{-SP} following MMP1 immunoprecipitation. C) MMP13 was overexpressed in 293T cells stably transduced with either a scrambled shRNA or an shRNA targeting VLK (#5). Tyrosine phosphorylation was examined in CM following MMP13 immunoprecipitation. VLK expression was determined in cell lysates (Lys.) D) Freshly isolated human platelets were treated with 0.5 μM TRAP for 15′. After centrifugation to remove cells, protein expression and tyrosine phosphorylation were determined in pellets and supernatants. E) Detergent free lysates of 293T cells expressing the indicated VLK constructs were treated with PK alone or in combination with NP40. See also Figure S4.

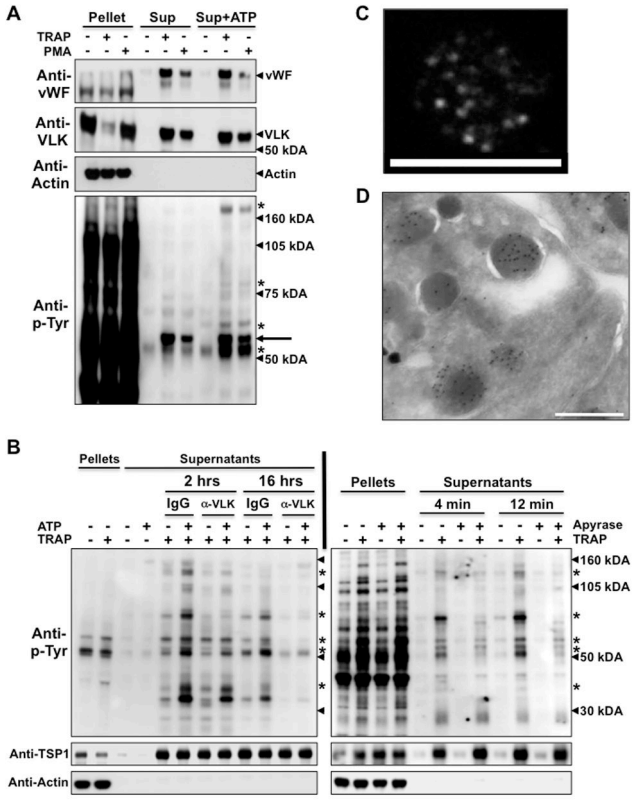

Figure 5. VLK expression enhances cellular and secreted tyrosine phosphorylation

A) K4 synoviocytes stably over-expressing GFP, VLK or VLK^{KM} were treated with 100 nM dasatinib for 6 hrs. Extent of tyrosine phosphorylation in lysates was assessed by immunoblotting. Asterisks indicate VLK-dependent phosphorylations. B) Tyrosine phosphorylation was detected in TCA-precipitated CM of K4 synoviocytes over-expressing VLK or VLK^{KM}. See also Figure S5.

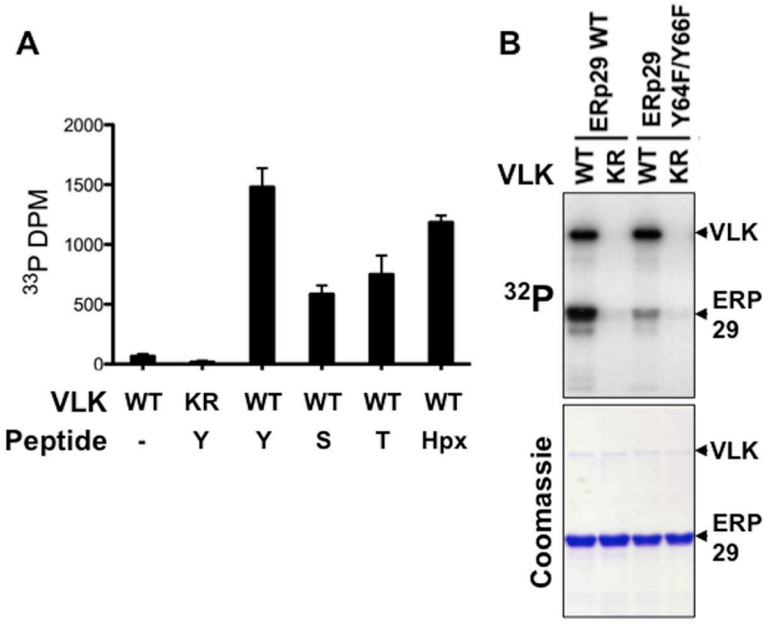

Figure 6. Baculovirus expressed VLK phosphorylates peptides and proteins

Wild type VLK, or VLK mutated at Lys66 (VLK^{KR}) was purified from CM of a baculovirus expression system and tested for peptide or protein phosphorylation activity. A) Biotinylated peptides were incubated with purified VLK protein in the presence of ^{33}[P]-γ-ATP, captured on streptavidin and counted by liquid scintillation counting. YPeptide: GRRYLQELQKEQ; SPeptide:GRRSLQELQKEQ, TPeptide GRRTLQELQKEQ; HpxPeptide: peptide derived from MMP1 hemopexin domain, QNVLHGYPKDI. B) Purified wild type or mutant recombinant ERP29 phosphorylated by purified recombinant wt VLK or VLK mutated at Lys166 (KR) analyzed by Laemmli gel electrophoresis and autoradiography. See also Figure S6.

Table I

Identification of tyrosine phosphopeptides in secreted proteins and extracellular domains in VLK expressing cells

Total tyrosine phosphopeptides were isolated by anti-pTyr immunoprecipitation of tryptic digests of cell lysates or CM and analyzed by LC-MS/MS. 140 unique phosphopeptides were identified, of which 48 (listed here) were mapped to proteins with canonical signal peptides, or extracellular domains of transmembrane proteins, as annotated in UniprotKB. Peptides identified exclusively in conditioned medium are shaded in bold. Tyrosine phosphorylation sites reported to occur in vivo in Phosphosite are marked with a “Y”, in parentheses the number of separate studies is shown. “TC” designates sites that were detected in only in tissue culture cell lines. See also Table S1 and Supplementary File 1.

Protein name	Positions within protein	Endogenous phosphorylation reported
Mesencephalic astrocyte-derived neurotrophic factor	76	Y(21)
Collagen alpha-1(I) chain	1215; 1216	
collagen prolyl 4-hydroxylase beta (P4HB)	94; 268	Y(1)
Prolyl 4-hydroxylase subunit alpha-2	79	Y(1 TC)
Osteopontin	165	
Follistatin-related protein 1	286;251	
Insulin-like growth factor-binding protein 3	190	
Insulin-like growth factor-binding protein 10	221	
Insulin-like growth factor-binding protein 7	201	
Protein kinase domain-containing protein, cytoplasmic (VLK)	64	
Alpha-1,6-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase	301	
Peroxiredoxin-4	266	
Lysozyme-like protein 1	106	
Serine protease 23	131	Y(1 TC)
Melanoma inhibitory activity protein 3	969	
Golgi integral membrane protein 4	407;379	Y (9)
Polypeptide N-acetylgalactosaminyltransferase 5	68	
Proadrenomedullin	52	
Secretogranin-2	457	Y(12)
Endoplasmic reticulum resident protein 44	395	
Cysteine-rich with EGF-like domain protein 2	68;	
Protein disulfide-isomerase A3	100; 445	
Endoplasmic reticulum resident protein 29	64; 66	Y(15);Y(365)
HLA class I histocompatibility antigen	31; 195	
Nucleobindin-1	179	Y(11)
Nucleobindin-2	169	Y(32)
reticulocalbin-1	115,190	Y(7)
Protein canopy homolog 2	71	
Protein canopy homolog 3	110	
Multiple inositol polyphosphate phosphatase 1	46	
Transmembrane emp24 domain-containing protein 10	135	

Protein name	**Positions within protein**	**Endogenous phosphorylation reported**
CRELD2	68	
Retina-specific copper amine oxidase	488	Y(1 TC)
prosaposin	187	
Galectin-3-binding protein	446	
78 kDa glucose-regulated protein (HSPA5)	65; 635	
Alpha-2-macroglobulin receptor-associated protein	336	Y(1)
Calumenin	275	Y(60)
Angiopoietin-related protein 5	165	
Amyloid beta A4 protein	588	
SorCS2	746; 574	
Epsilon-sarcoglycan	38	Y(1 TC)
Group XIIA secretory phospholipase A2	130	
Neutral alpha-glucosidase AB	363	Y(1)
MESD	58	