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VITAMIN K_1-DEPENDENT GROWTH REGULATORY PATHWAYS DURING EMBRYOGENESIS

by

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ABSTRACT

The normal embryonic development requires a well coordinated repertoire of cellular activities including cell division, differentiation, and migration. Protein tyrosine kinases (PTKs) are involved in regulating these events during embryonic development. Recently, vitamin K-dependent γ-carboxylated proteins and Gas6 have been identified as ligands for a unique family (Tyro 3, 7 and 12) of receptor tyrosine kinases (RTKs). Since Tyro 7 and 12 RTKs show wide spatial and temporal expression during embryonic development, and overexpression of Axl (Tyro 7) in transgenic mice appears to cause prenatal lethality, the developmental signals from vitamin K-dependent receptor-ligand system are required for orderly embryogenesis. Furthermore, the involvement of vitamin K metabolism and functions in two well characterized birth defects, warfarin embryopathy and vitamin K epoxide reductase deficiency, also supports this hypothesis. Using a chick model of embryogenesis, we demonstrated the existence of a vitamin K_1-dependent protein-tyrosine phosphorylation cascade during embryogenesis. This cascade is sensitive to alteration in levels and metabolism of vitamin K_1 and involves c-Eyk, a member of the Tyro 12 family, and a group of key intracellular proteins, including focal adhesion kinase ($pp125^{FAK}$), paxillin, and $pp60^{c\text{-}src}$. The precise regulation of vitamin K_1-dependent regulatory pathways appears to be critical for orderly embryogenesis. These findings explain partly why, in the mammalian fetus, the vitamin K-dependent proteins are maintained in an undercarboxylated state, even to the point of placing the newborn at hemorrhagic risk.

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LIST OF FIGURES

LIST OF ABBREVIATIONS

min	minute
h	hour
°C	degree Celsius
mL	milliliter
ng	nanogram
µg	microgram
mg	milligram
µM	micromolar
mM	millimolar
%	percent
kDa	kilodalton
anti-PY	anti-phosphotyrosine
BSA	bovine serum albumin
Csk	C-terminal Src kinase
ddH_2O	distilled deionized water
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene-diamine-tetraacetic-acid
FAT	focal adhesion targeting
Gla	carboxyglutamic acid

Glu	glutamate
Graf	GTPase regulator associated with $pp125^{FAK}$
GRB2	growth factor receptor-binding protein 2
JAK	Janus kinase
LIM domain	lin-11 isl-1 mec-3 domain
MBP	myelin basic protein
MW	molecular weight
$p34^{cdc2}$	cell division cycle gene product
$P130^{cas}$	Crk-associated substrate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PH domain	pleckstrin homology domain
PI	phosphatidylinositol
PI 3-kinase	phosphatidylinositol 3-kinase
PKB	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
$pp125^{FAK}$	focal adhesion kinase
PTKs	protein tyrosine kinases
PTPases	protein tyrosine phosphatases
RIPA buffer	radioimmunoprecipitation assay buffer
RSV	Rous sarcoma virus

RTKs	receptor tyrosine kinases
SDS	sodium dodecyl sulphate
SH-2 domain	Src homology 2 domain
SH-3 domain	Src homology 3 domain
SOS	son of sevenless
STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline

INTRODUCTION

Vitamin K_1 (2-methyl-3-phytyl-1,4-napthoquinone) is a micronutrient whose known function is to act as a cofactor for the carboxylase involved in post-translational carboxylation of a series of glutamic acid (Glu) residues in juxtaposition to the N-terminus of the vitamin K-dependent proteins (Vermeer *et al.*, 1995). Vitamin K_1 once thought to only play a role in the hepatic synthesis of just four procoagulant proteins (prothrombin or factor II and factor VII, IX and X), is now involved in the γ-carboxylation of two coagulation inhibitors (protein C and S), osteocalcin, matrix carboxyglutamic acid (Gla) protein of bone, as well as several other proteins of unknown functions (Furie and Furie, 1990). Recent studies demonstrating the presence of vitamin K-dependent proteins as ligands for the receptor tyrosine kinases (RTKs) that can drive cellular proliferation and transformation, identified a previously unrecognized and potentially important role for vitamin K in growth regulation (Varnum *et al.*, 1995).

I. VITAMIN K_1

1. STRUCTURE OF VITAMIN K_1

Vitamin K, a fat soluble vitamin essential for the production of prothrombin and other coagulation factors by liver, was discovered by Henrik Dam in 1929 in studies of chicks fed fat-free diets (Olson, 1984). Vitamin K_1, known as phylloquinone, is the only vitamin K homologue present in plants. The structure of vitamin K_1 is shown in Figure 1 (MacCoquodale *et al.*, 1939).

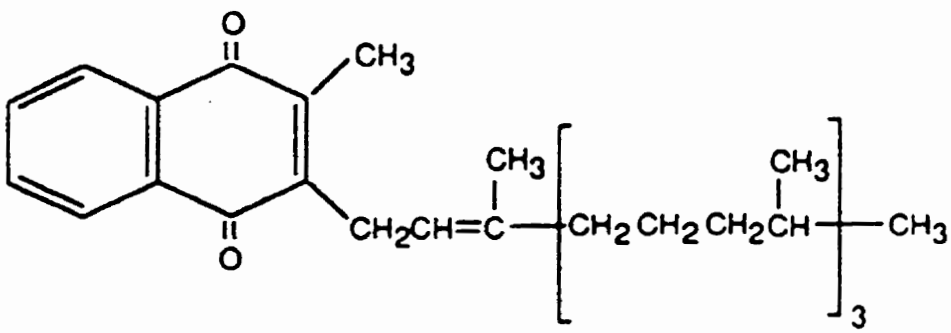

Figure 1. Structure of Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone).

2. SOURCES AND REQUIREMENTS OF VITAMIN K_1

Vitamin K is derived both from foods and the microflora of the gut (Olson, 1984). Phylloquinone (vitamin K_1) and menaquinone (vitamin K_2) are most abundant forms of vitamin K. Although the amount of vitamin K_1 in food varies with soil and growth conditions, geographical differences, and time of harvesting, in general, the following approximate ranges may be given (Booth *et al.*, 1993):

green leafy vegetables	1000 ~ 8000 μg/Kg
other vegetables and fruits	10 ~ 500 μg/Kg
dairy produce	3 ~ 70 μg/Kg
grains	0.5 ~ 70 μg/Kg

In green vegetables, vitamin K_1 is tightly bound to the thylakoid membranes of the chloroplasts (Lichtenthaler, 1993). The gastrointestinal extraction from green vegetables is less efficient than that from foods like dairy produce in which the vitamin K_1 is solubilized in the fat component, where it may be absorbed without membrane degradation (Vermeer *et al.*, 1995). The intestinal absorption of vitamin K_1 from plant sources ranges from 30 % to 70 % of the actual content determined by extraction (Olson, 1984). Furthermore, the efficacy of vitamin K_1 absorption from the intestinal lumen depends on the stimulation of secretion of bile salts and pancreas lipase by ingested fats (Vermeer, 1995).

The human hepatic storage pool of vitamin K_1 in adult liver ranged between 1.7 to 38.3 μg (median, 7.8 μg. Shearer *et al.*, 1988). The minimal daily requirement for vitamin K_1 in adults is between 0.03 to 1.5 ng/Kg body weight daily. This amount approximates to

the size of the hepatic pool in the adult (Frick *et al.*, 1967). In the elderly, vitamin K_1 deficit is common and has been suggested to be a significant factor in osteoporosis and hip fractures. Although in the adult the plasma levels of vitamin K_1 are higher than in the newborn, the storage pool of vitamin K_1 is surprisingly small. In healthy young adults, restriction dietary vitamin K_1 intake to 10 μg/day for 13 days resulted in a dramatic decrease in the plasma vitamin K_1 levels below the normal of 0.29 ~ 2.64 nM within 7 days (Sadowski *et al.*, 1989). These evidence suggest that there is no significant vitamin K storage pool in body, and humans are dependent on a continual dietary supply of vitamin K. The vitamin K requirement of mammals is met by a combination of dietary intake and microbiologic biosynthesis in the gut. In humans, about 40 ~ 50 % of the daily vitamin K requirement is derived from plant sources and the remainder from microbiologic biosynthesis (Olson, 1984). Both phylloquinone and menaquinone are present in human plasma. The plasma phylloquinone is present in the range of 0.5 ~ 5.0 ng/mL with an average of 2 ng/mL in healthy persons. Menaquinone is present in lower amounts (Chiu *et al.*, 1981; Lefevere *et al.*, 1979; Shearer *et al.*, 1982). A "normal mixed diet" in the United States contains 300-500 μg vitamin K per day, which is more than adequate to supply the dietary requirement of vitamin K (Olson, 1984).

3. METABOLISM OF VITAMIN K_1

In the hepatocyte, vitamin K is reduced to the vitamin K hydroquinone (vitamin KH_2) by vitamin K reductase (an enzyme sensitive to inhibition by warfarin. Suttie, 1985). Vitamin KH_2 is a cofactor in the reaction, catalyzed by a vitamin K-dependent carboxylase, in which Glu residues of the precursor form of a vitamin K-dependent protein

are modified to γ-carboxyglutamic acid (Gla). This reaction still needs O_2 and CO_2. After this reaction, Gla residues are generated and vitamin KH_2 is converted to vitamin K epoxide. Then the vitamin K epoxide is converted back to vitamin K by the vitamin K epoxide reductase (another enzyme sensitive to inhibition by warfarin. Whitlon *et al.*, 1978).

4. FUNCTION OF VITAMIN K_1

Vitamin K_1 functions in the post-translational modification of liver microsomal protein precursors to form biologically active prothrombin, factor IX, factor X, factor VII, protein C and protein S (Furie and Furie, 1990). This modification involves the carboxylation of specific Glu residues in the precursor proteins to form Gla residues in these proteins (Stenflo *et al.*, 1977. Figure 2).

Vitamin K epoxide reductase is inhibited by warfarin. So vitamin K-vitamin-2,3-epoxide cycle is blocked. There is evidence to indicate that warfarin exerts its effect on prothrombin synthesis through its action on vitamin K epoxide reductase (Willingham *et al.*, 1974).

The active form of vitamin K in the carboxylase system is vitamin KH_2. Vitamin K reductase can convert vitamin K to vitamin KH_2. This enzyme is also sensitive to warfarin inhibition (Furie and Furie, 1990).

Carboxylation of Glu in the vitamin K-dependent zymogen precursors to the enzymes of the blood-clotting cascade is a post-translational event that occurs at the N-terminus of the nascent chain. Carboxylation converts the selected Glu in the clotting-cascade proteins to Gla residues to enable the proteins to bind calcium. The bound calcium

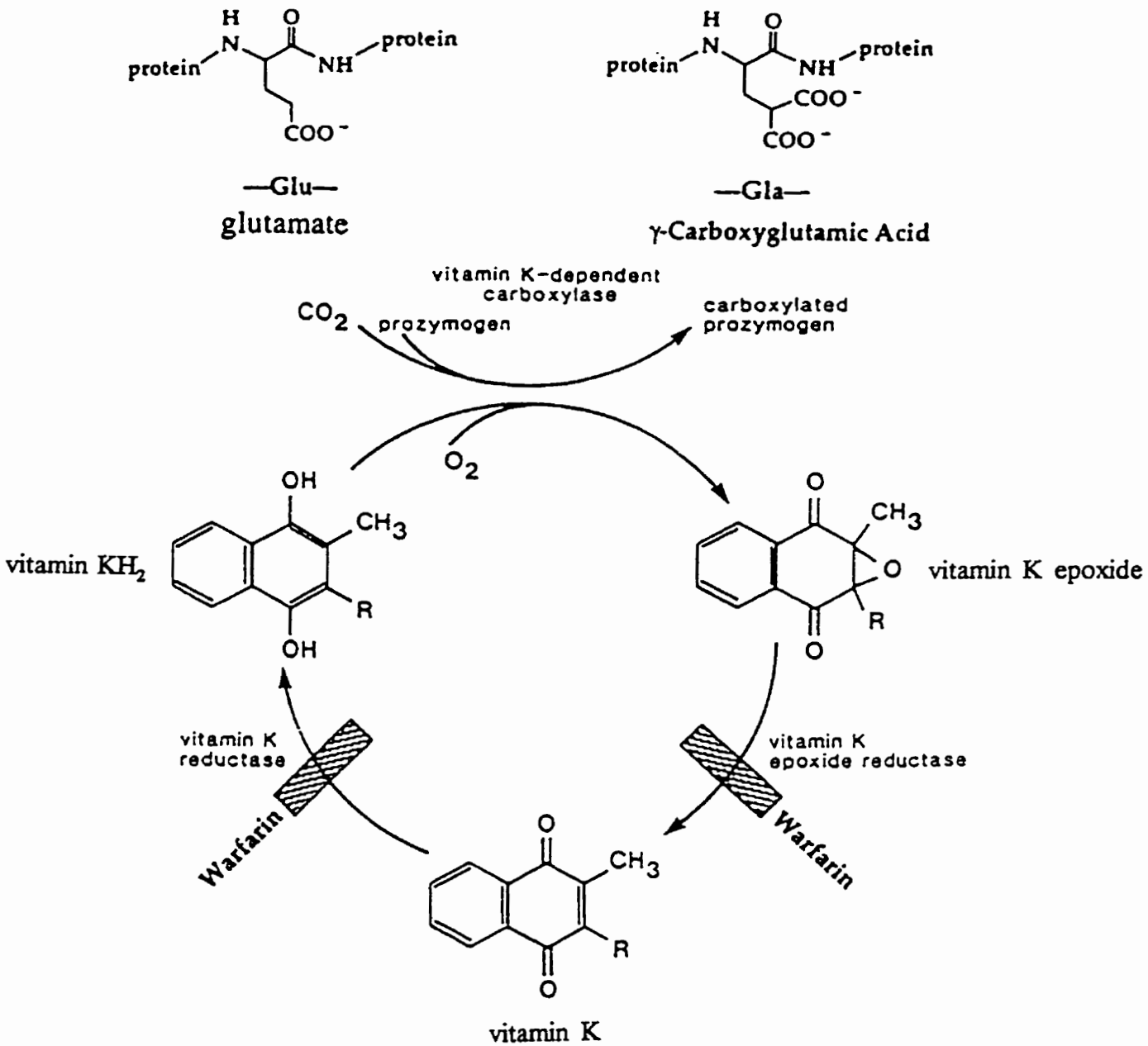

Figure 2. Vitamin K-Vitamin-2,3-Epoxide Cycle. Vitamin K is reduced to the vitamin K hydroquinone (vitamin KH_2) by vitamin K reductase, vitamin KH_2 is the substrate for the vitamin K-dependent carboxylase (vitamin K epoxidase). With the carboxylation of Glu residues on the protein substrate, CO_2 and O_2, vitamin K epoxide is formed. The vitamin K epoxide is cycled back to vitamin K by the vitamin K epoxide reductase. Warfarin can inhibit vitamin K epoxide reductase and vitamin K reductase (Furie and Furie, 1990; Dowd et al., 1995).

forms an ion bridge between the blood-clotting enzymes and phospholipids on the membrane surfaces of blood platelets and endothelial and vascular cells. Calcium binding also plays an essential role in controlling coagulation protein conformation by enabling internal Gla-Gla binding (Cain *et al.*, 1990; Lewis *et al.*, 1988; Pollock *et al.*, 1988; Soriano-Garcia *et al.*, 1989). Calcium binding also provides the rationale for carboxylation of glutamate residues in the bone proteins osteocalcin and matrix Gla protein (Hauschka *et al.*, 1975; Hauschka *et al.*, 1978). Transport of Ca^{2+} by the chick chorioallantoic membrane from the egg shell to the embryo was recently shown to be vitamin K dependent. This capacity to transport Ca^{2+} developed in parallel with bone mineralization implicates vitamin K in the mineralization process (Gijsbers *et al.*, 1990; Lian and Friedman, 1978).

Recent evidence supports the presence of vitamin K-dependent proteins as ligands for the RTKs that can regulate cellular proliferation and transformation (Varnum *et al.*, 1995). This demonstrates that vitamin K may play a potentially important role in growth regulation.

II. RECEPTOR TYROSINE KINASE (RTKs)

Protein-tyrosine kinases (PTKs) can be divided into two general categories: receptor tyrosine kinases (RTKs) and cytoplasmic tyrosine kinases, including members of the *src*, *fps*, and *abl* gene families (Hanks *et al.*, 1988). RTKs form an important class of cell surface receptors with intrinsic protein-tyrosine kinase activity (Schlessinger and Ullrich, 1992). In the presence of the appropriate ligand, RTKs trigger a receptor's

intrinsic tyrosine kinase activity and lead to auto-phosphorylation, thereby transducing an external signal to the inside of the cell (Schlessinger, 1988). RTKs have been shown to play a central role in transducing the external signals across cell membranes into intracellular signaling systems and these signals lead to cell proliferation, differentiation, and other responses (Ullrich *et al.*, 1990).

Recently, extensive sequence similarity shared by tyrosine kinase domains has allowed for homology-based cloning of a large number of proteins that appear to be RTKs, in that they are predicted transmembrane proteins with large ectodomains and a cytoplasmic tyrosine kinase domain (Lai and Lemke, 1991). These proteins have been designated as orphan RTKs because their presumed ligands have yet to be identified. These RTKs, designated as Tyro 3, Tyro 7, and Tyro 12, display structural similarities but differential patterns of tissue expression.

Tyro 3 is also named as Sky, rse, brt, or tif (Lai *et al.*, 1994; Ohashi *et al.*, 1994; Mark *et al.*, 1994; Fujimoto and Yamamoto, 1994; Dai *et al.*, 1994). It is most prominently expressed in the adult nervous system, and also highly expressed in kidney, ovary, testis and a number of hematopoietic cell lines.

Tyro 7 is also named as Axl, UFO, or Ark (O'Bryan *et al.*, 1991; Janssen *et al.*, 1991; Rescigno *et al.*, 1991; Bellosta *et al.*, 1995). It is expressed in the nervous system and peripheral tissues.

Tyro 12 is also named Eyk (chicken form. Jia and Hanafusa, 1994), or cMer (human form. Graham *et al.*, 1994). It is also expressed in nervous system and peripheral tissues. The structure of c-Eyk is shown in Figure 3.

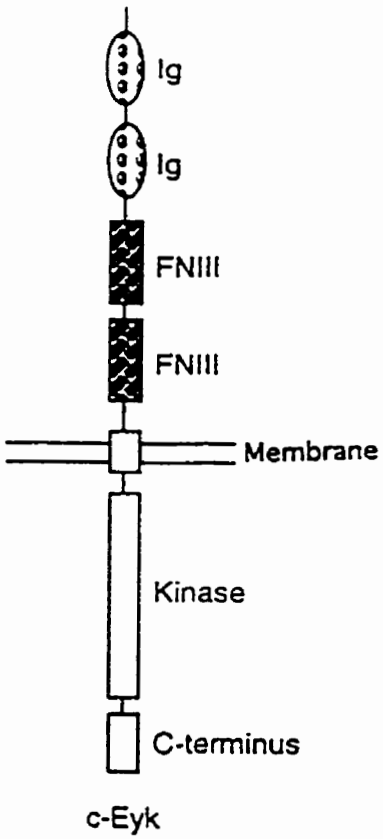

Figure 3. Schematic protein structure of c-Eyk. The horizontal double lines in the middle represents the plasma membrane, above it is the extracellular space and below it is the intracellular space.

III. VITAMIN K-DEPENDENT PROTEINS AS LIGANDS OF RTKs

Recently, protein S, a vitamin K-dependent coagulation inhibitor (Dahlback, 1991), and its relative Gas6, a product of growth-arrest specific gene 6, have been identified as classical ligands for Tyro 3, 7 and 12 family of RTKs (Stitt *et al.*, 1995; Godowski *et al.*, 1995; Ohashi *et al.*, 1995).

Protein S is a vitamin K-dependent protein with multiple domains. It can bind and activate Tyro 3 (Stitt *et al.*, 1995). From the N-terminal it contains a vitamin K-dependent domain, a thrombin-sensitive region, four epidermal growth factor (EGF)-like domains and C-terminal region homologous to the androgen binding proteins (Dahlback, 1991). Protein S functions as a non-enzymatic cofactor to activated protein C and promotes degradation of the coagulation factors Va and VIIIa, thus serves as anticoagulant activity (Dahback, 1991). Protein S has also been suggested to have other functions outside of clotting system. When protein S binds to C4b-binding protein, the function of protein S as an activated protein C cofactor is lost (Dahlback, 1991). The role of protein S as a mitogen for smooth muscle cell is also suggested(Gasic *et al.*, 1992). The production of protein S by bone cell or neural tumor cell lines indicates that protein S might have some functions in the bone and brain (Maillard *et al.*, 1992; Phillips *et al.*, 1993).

Gas6 has recently been identified as a ligand for Sky, Axl and Mer RTKs (Godowski *et al.*, 1995; Ohashi *et al.*, 1995; Varnum *et al.*, 1995; Chen *et al.*, 1997). Gas6 manifests 42 ~ 43 % amino acid identity and a similar domain organization with protein S (Manfioletti et al., 1993). The N-terminal Gla domain of Gas6 is rich in Gla

residues. Gla domains commonly serve to mediate the Ca^{2+} dependent binding of proteins to negatively charged phospholipids present in cell membranes. A loop region and four EGF-like repeats follow the Gla domain. The loop region of protein S contains thrombin-sensitive cleavage sites, although these are not conserved in Gas6. The C-terminal portions of Gas6 and protein S are similar to the steroid hormone binding globulin protein (Gershagen *et al.*, 1987; Hammond *et al.*, 1987) and contain G domains. G domains are present in numerous proteins involved in cell growth and differentiation (Joseph and Baker, 1992; Patthy and Nikolics, 1993). The G domains of Gas6 are sufficient to bind with high affinity to Rse or/and to Axl and can activate receptor phosphorylation with a specific activity similar to that of the full length molecule (Mark *et al.*, 1996). Vitamin K-dependent γ-carboxylation of Gas6 is required for its full activity (Varnum *et al.*, 1995). Gas6 has been reported to block apoptosis induced by growth arrest in rat vascular smooth muscle cells (Nakano *et al.*, 1996).

The role of this novel vitamin K-dependent receptor-ligand system in cellular processes is not clear but some studies have demonstrated the transforming activity of Axl in NIH 3T3 cells (O'Bryan *et al.*, 1991; McCloskey *et al.*, 1994). Overexpression of Axl and Sky led to cell transformation (O'Bryan *et al.*, 1991; Lai *et al.*, 1994; Taylor *et al.*, 1995). The near-ubiquitous expression and transforming activity of Axl suggest that this receptor can drive cellular proliferation (O'Bryan *et al.*, 1991; Varnum *et al.*, 1995). Furthermore, as the Axl/Sky family receptors have oncogenic potential, they may be involved in tumor progression and in normal cell proliferation. The involvement of vitamin K metabolism and functions in two well characterized birth defects, warfarin embryopathy

(Hall *et al.*, 1980) and vitamin K epoxide reductase deficiency (Pauli *et al.*, 1987), suggests that developmental signals from vitamin K-dependent pathways may be required for normal embryogenesis. All together, the vitamin K-dependent receptor-ligand system may play a role in growth regulation during embryogenesis.

IV. pp125^{FAK}, Paxillin, pp60$^{c\text{-}src}$, c-Eyk, and PI 3-Kinase

1. Focal adhesion kinase (pp125^{FAK})

The changes in cytoskeletal structure are crucial to a number of cellular events associated with cell growth, migration, and division. pp125^{FAK} , originally isolated in v-src-transformed chicken embryo fibroblasts, is a prominent tyrosine phosphorylated protein (Schaller *et al.*, 1992). It is found at cellular focal adhesions, co-localized with a number of other cytoskeletal proteins such as talin and paxillin (Kornberg *et al.*, 1992). Phosphorylation of those proteins is downstream of pp125^{FAK} activation (Seufferlein and Rozengurt, 1995; Rankin and Rozengurt, 1994), suggesting that this tyrosine kinase plays a role in regulating cytoskeletal assembly.

Cloning studies on pp125^{FAK} show little homology with other tyrosine kinases, no acylation and an absence of both SH-2 and SH-3 domains (Schaller *et al.*, 1992). It has a central catalytic domain flanked by large N-and C-terminal domains. Sequences within the C-terminus of pp125^{FAK} regulate its localization to focal adhesions and are also required for binding the cytoskeletal protein, paxillin (Hildebrand *et al.*, 1993; Schaller and Parsons, 1994). Other structural domains in pp125^{FAK} provide putative binding sites for a variety of other signaling molecules, such as PI 3-kinase, growth factor receptor-binding

proteon 2 (GRB2), Src-family kinase, Crk-associated substrate ($P130^{cas}$), GTPase regulator associated with FAK (Graf) and structural proteins such as β-integrin and talin. It is known that autophosphorylation of $pp125^{FAK}$ on Tyr-397 generates an SH-2 mediated interaction with $pp60^{c\text{-}src}$ (Schaller *et al.*, 1994. Figure 4). This interaction enzymatically activates the Src family kinase which, in turn, phosphorylates Tyr-407, Tyr-576, and Tyr-577 of $pp125^{FAK}$ to fully activate this kinase (Calalb *et al.*, 1995).

2. Paxillin

Paxillin, a 68-kDa protein, localized to the focal adhesions at the ends of actin stress fibers. It is potentially involved in actin-membrane attachment at focal adhesions via an interaction with vinculin (Turner *et al.*, 1990). It is a protein of multiple isoforms with pIs ranging from 6.31 to 6.85.

A full-length cDNA encoding human paxillin was cloned, revealing multiple protein domains, including four tandem LIM domains (a cysteine-rich motif: $C\text{-}X_2\text{-}C\text{-}X_{17\text{-}19}\text{-}H\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_{7\text{-}11}\text{-}(C)\text{-}X_8\text{-}C$. It is present in the proteins encoded by the genes *lin-11*, *isl-1* and *mec-3*, refered to as LIM for *lin-11 isl-1 mec-3*. Freyd *et al.*, 1990), a proline-rich domain containing a consensus SH-3 binding sites. The paxillin gene was localized to chromosome 12q24 by fluorescence in situ hybridization analysis. It has been shown to have binding sites for the SH-3 domain of Src and the SH-2 domain of Crk (encoded by an oncogene *v-crk*, a transforming gene discovered in avian retrovirus genome of CT10. Mayer *et al.*, 1988) in vitro and to coprecipitate with two other focal adhesion proteins, vinculin and $pp125^{FAK}$. Binding of v-Src and v-Crk to paxillin may be a major determinant in concentrating both of these oncogenes in focal adhesions and therefore could be

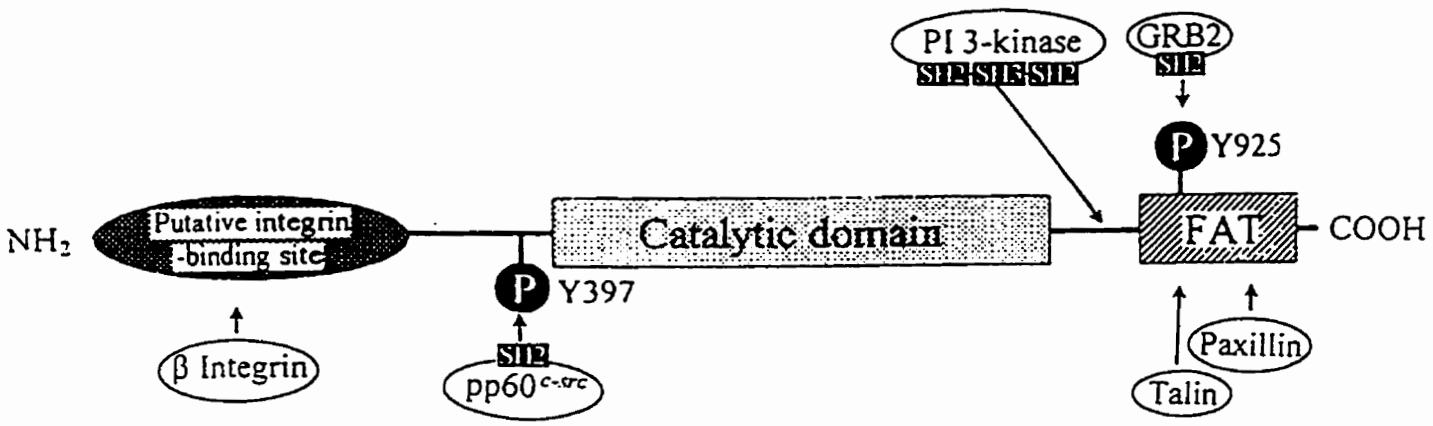

Figure 4. Sites of interaction for pp125^{FAK}-binding proteins. Phosphorylation-dependent binding sites are present on either side of the catalytic domain pp125^{FAK} and allow complex formation with the SH-2 domains of the pp60$^{c\text{-}src}$ and GRB2. Binding sites in the N-and C-terminal tails are required for pp125^{FAK} binding to cytoskeletal proteins, such as β-subunit of integrins, talin and paxillin. Proline-rich sequences within the C-terminal domain provide potential binding sites for the SH-3 domain of PI 3-kinase.

important in the altered cytoskeletal structure and adhesive properties that accompany transformation by both viral oncogenes (Salgia *et al.*, 1995).

Paxillin is heavily phosphorylated on tyrosine during cell transformation, it also contains high levels of phosphoserine and phosphothreonine. Paxillin may serve as an adapter protein itself, a protein that tethers other proteins to a multicomponent complex. Tyrosine phosphorylation of paxillin may function to regulate such protein-protein interactions. Paxillin colocalized with $pp125^{FAK}$ in cellular focal adhesions. $pp125^{FAK}$ and paxillin form a stable complex *in vivo* and thus are inclose enough proximity to function as enzyme and substrate. It is been proposed that the activation of $pp125^{FAK}$ leads to the phosphorylation of paxillin on tyrosine, either directly or via a second intermediate PTK, like $pp60^{src}$, that is activated by $pp125^{FAK}$. Phosphorylation of paxillin creates binding sites for the SH-2 domains of signalling molecules such as C-terminal Src kinase (Csk) and the adapter protein Crk. Crk contains a single SH-2 and two SH-3 domains and through these latter motifs complexes with C3G and SOS, two guanine nucleotide exchange proteins that can drive the conversion of inactive $p21^{ras}$-GDP into active $p21^{ras}$-GTP (Matsuda *et al.*, 1994; ten Hoeve *et al.*, 1993). Through this mechanism, paxillin may play a key role in the regulation of the activation of GTP-binding proteins in a very specific location within the cell. Once active, the GTP-binding proteins could transmit a signal to the nucleus and / or a signal inducing structural changes to the cytoskeleton (Schaller and Parsons, 1995).

Paxillin has been identified as one of the major targets for tyrosine kinases in a variety of tissues during chick embryonic development. The change in the level of tyrosine

phosphorylation of paxillin during the chick embryo development suggests that tyrosine phosphorylation of paxillin may regulate the formation of stable actin-membrane interactions required for normal organogenesis and adult organ function (Turner, 1991).

3. $pp60^{c\text{-}src}$

The Src proteins were the first tyrosine kinases to be discovered. $pp60^{c\text{-}src}$ is a non-receptor tyrosine kinase that resides within the cell associated with cell membranes and appears to transduce signals from transmembrane receptors to the interior of the cell. Many intracellular pathways can be stimulated by $pp60^{c\text{-}src}$ activation, leading to a variety of cellular consequences, including morphological changes and cell proliferation. $pp60^{c\text{-}src}$ is the normal cellular homologue of the Rous sarcoma virus (RSV) gene product $pp60^{v\text{-}src}$. It shows a restricted tissue distribution and is likely to participate in specific interactions with upstream and downstream signaling intermediates. Its highest level of expression is in neural tissues (Cotton and Brugge, 1983; Levy *et al.*, 1984; Sorge *et al.*, 1984). It is particularly concentrated in the nerve growth cones (Maness *et al.*, 1988) where tubulin has been shown to be one of its substrates (Matten *et al.*, 1990). It contains an N-terminal myristoylation signal, SH-2 and SH-3 domains, a catalytic site and a C-terminal autoregulatory tail (Superti-Furga and Courtneidge, 1995). SH-2 domains are highly conserved regions of approximately 100 amino acids which recognize specific consensus sequences encompassing tyrosine phosphorylated residues, whereas SH-3 domains which consist of approximately 60 amino acids, bind specific proline-rich sequences (Pawson and Schlessinger, 1993). A N-terminal glycine residue which

undergoes myristoylation is responsible for localizing $pp60^{c\text{-}src}$ to cellular membranes (Cross *et al.*, 1985). C-terminal tyrosine residue plays an important role in $pp60^{c\text{-}src}$ autoregulatory (Bagrodia *et al.*, 1991).

The activity of $pp60^{c\text{-}src}$ is thought to be predominantly controlled by a tyrosine phosphorylation site in its C-terminal tail at residues Tyr 530 in human $pp60^{c\text{-}src}$ or Tyr 527 in chicken $pp60^{c\text{-}src}$ (Tanaka and Fujita, 1986; Partanen *et al.*, 1987; Kato *et al.*, 1987; Cartwright *et al.*, 1987; Piwnica-Worms *et al.*, 1987; Kmiecik and Shalloway, 1987; Cooper *et al.*, 1986; Laudano and Buchanan, 1986). This site can be phosphorylated by an enzyme known as Csk, resulting in $pp60^{c\text{-}src}$ inactivation due to an intramolecular interaction between the phosphotyrosine in the C-terminal tail and a domain within the N-terminal half of the molecule known as the SH-2 domain (Liu *et al.*, 1993; Bibbins *et al.*, 1993; Murphy *et al.*, 1993; Okada *et al.*, 1993; Superti-Furga *et al.*, 1993; Roussel *et al.*, 1991). Tyr 527, once phosphorylated, then becomes a substrate for protein tyrosine phosphatases. Under normal conditions, the level of $pp60^{c\text{-}src}$ tyrosine kinase activity is probably regulated by a combination of both negative regulatory effects of Csk phosphorylation of Tyr 527 as well as the activating effect of C-terminal protein tyrosine phosphatase activities.

The localization of $pp60^{c\text{-}src}$ to the cytoskeleton is associated with the tyrosine phosphorylation of a number of cytoskeletal proteins, including paxillin, talin, vinculin, $pp125^{FAK}$ and the cytoplasmic domain of β1-integrin (Burridge *et al.*, 1992; Findik *et al.*, 1990). Increasing evidence indicates that the activation and subcellular localization of

pp60$^{c\text{-}src}$ are tightly coordinated processes critically dependent on the tyrosine phosphorylation status of the C-terminal tyrosine residue-527 (Kaplan *et al.*, 1995). Dephosphorylation of this residue by cellular phosphatase(s) is proposed to lead to a conformational change in the enzyme's structure, exposing N-terminal domains required for focal adhesion localization.

The amount of pp60$^{c\text{-}src}$ encoded by *c-src* was found to change during embryonic development and to vary from tissue to tissue, with the highest levels in brain and other neural tissues (Jacobs and Rubsamen, 1983; Cotton and Brugge, 1983; Schartl and Barnekow, 1984; Levy *et al.*, 1984), suggesting that pp60$^{c\text{-}src}$ may play a role in neuronal development.

4. c-Eyk

The chicken version of Tyro 12 has been called c-Eyk (East Lansing Tyrosine Kinase, East Lansing is the place where the RPL30 virus was originally isolated. Jia and Hanafusa, 1994). The proto-oncogene *c-eyk*, from which *v-eyk* (a viral oncogene from the RPL30 virus) was derived, codes for an RTK (c-Eyk) with a distinctive extracellular region, consisting of two C2-type immunoglobulin-like loops and two fibronectin-III repeats, which makes this molecule very unique (Figure 3). The presence of Ig/FN-III domains in a PTK may allow kinases to respond to cell surface ligands; this direct interaction will impose short range regulation to complex cell growth and differentiation control. *c-eyk* expression is tighly regulated in chicken cells, it in general is inactive and not transforming, but v-Eyk kinase is constitutively active (Zong *et al.*, 1996). But so far, the ligand of c-Eyk has still not been identified although Chen and co-workers reported

that the protein Gas6 serves as a ligand for Mer (a human analog of c-Eyk. Chen *et al.*, 1997). Another group reported that activated c-Eyk can constitutively activate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, specifically JAK1, STAT1 and STAT3, and that c-Eyk activity is essential for STAT1 activation, which correlates well with cellular transformation (Zong *et al.*, 1996).

Studies of expression patterns of *c-eyk* showed that *c-eyk* is expressed in chicken embryonic liver, heart, muscle, brain, and chicken embryo fibroblast. c-Eyk is also observed in chicken embryonic liver, intestine, and lung in all four developmental stages (chicken embryonic day 12 and day 20 as well as postnatal day 2 and day 12). This evidence indicates that the *c-eyk* gene is turned on in the all chicken embryonic developmental stages, stays on throughout the embryonic developmental process, and is still expressed after birth into the adult stage (Jia and Hanafusa, 1994). This wide expression pattern of *c-eyk* both spatially and temporally in the developmental process suggested that *c-eyk* may be required to transmit developmental signals in this process.

5. PI 3-kinase

PI 3-kinase activity was discovered by virtue of its physical association with the activated platelet-derived growth factor (PDGF) receptor and the polyoma middle t/ $pp60^{src}$ complex (Kaplan *et al.*, 1986; Whitman *et al.*, 1988). When immunoprecipitated by anti-phosphotyrosine (anti-PY) antibodies, these activated complexes showed increased polyphosphoinositide lipid kinase activity which specifically phosphorylated phosphatidylinositol (PI) and other polyphosphoinositides (PI 4-P and PI 4,5-P_2) on position 3' of the inositol ring (Whitman *et al.*, 1988. Figure 5).

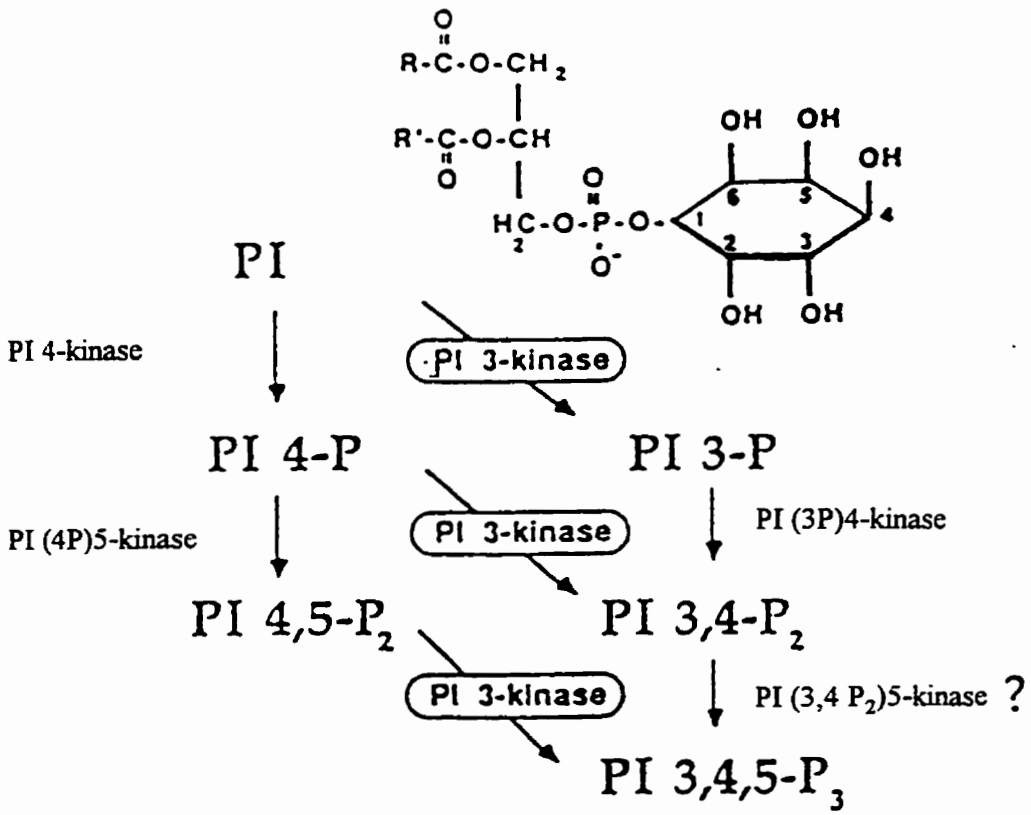

Figure 5. Generation of 3' phosphorylated polyphosphoinositides from PI, PI 4-P and PI 4,5-P_2. The upper panel shows the structure of phosphatidylinositol (PI). The numbers on the inositol ring indicate positions of hydroxyl groups (Varticovski *et al.*, 1994).

PI 3-kinase consists of regulatory and catalytic subunits, 85 and 110 kDa, respectively. The 85 subunit of PI 3-kinase (p85) contains several specific domains of homology to known proteins: one SH-3, two SH-2, and a BCR-like domain (Varticovski *et al.*, 1994). It is the regulatory subunit for the catalytic subunit (p110) and itself has no catalytic activity (Escobedo *et al.*, 1991; Otsu *et al.*, 1991). The region corresponding to the sequences between the two SH-2 domains of p85 is sufficient for association with p110 (Hu *et al.*, 1993; Klippel *et al.*, 1993). Expression of the catalytic p110 subunit results in catalytic activity only in insect cells or when co-expressed with p85 in mammalian cells (Hiles *et al.*, 1992).

PI 3-kinase is a key component of tyrosine kinase regulated signaling pathways that lead to cell growth. It phosphorylates phosphoinositides at the D3 hydroxyl of inositol, producing phosphatidylinositol (3)-phosphate (PI 3-P), phosphatidylinositol (3,4)-bisphosphate (PI 3,4-P_2), phosphatidylinositol (3,4,5)-trisphosphate (PI 3,4,5-P_3. de Camilli *et al.*, 1996). PI 3-kinase products have independent roles as second messenger in complex cellular events. There a evidence that the pleckstrin homology (PH) domains of protein kinase B (PKB) is able to bind PI 3,4,5-P_3 (James *et al.*, 1996). PI 3,4,5-P_3 may play a dual role in the activation of PKB: binding directly to its PH domain and allowing its phosphorylation and consequent activation by an upstream, also PI 3,4,5-P_3-sensitive, unknown protein kinase (Stokoe *et al.*, 1997). A number of cellular processes require PI 3-kinase activity, including mitogenesis (Cantley *et al.*, 1991), membrane ruffling (Kotani *et al.*, 1994; Wennstrom *et al.*, 1994), fluid-phase pinocytosis (Baker *et al.*, 1995; Clague

et al., 1995), the respiratory burst (Baggiolini *et al.*, 1987; Ninomiya *et al.*, 1994), and lysosomal enzyme sorting (Brown *et al.*, 1995; Davidson *et al.*, 1995; Schu *et al.*, 1993).

V. PROTEIN-TYROSINE PHOSPHATASES (PTPases)

The phosphorylation of proteins in tyrosine residues plays a critical role in cellular processes such as differentiation, signal transduction and transformation (Krebs and Beavo, 1979). The interconversion of proteins between phosphorylated and nonphosphorylated forms is recognized as one of the most prevalent mechanisms for the reversible modulation of enzyme activity (Cohen, 1982). Actually, the intracellular levels of phosphotyrosine proteins are regulated by interaction between PTKs and their antagonists, protein-tyrosine phosphatases (PTPases). PTPases attenuate the activity of PTKs by dephosphorylating cellular target proteins which may be involved in regulatory cellular events. The balance of PTPase and PTK activity ensures normal cell growth properties.

The first report of protein tyrosine phosphorylation came from evidence showing that v-Src (the transforming principle of the Rous sarcoma virus) had tyrosine kinase activity (Hunter and Sefton, 1980; Levinson *et al* ., 1980). This led to an analysis of the cellular phosphoamino acid content, which revealed that 0.01 to 0.05 % was present as phosphotyrosine (Hunter and Sefton, 1980). In contrast to the rapid characterization of many PTKs, little is known about the structure and regulation of the PTPases. The major limitations are the difficulty in isolating tyrosine phosphatase as well as selecting and preparing suitable substrates. The sequence of the PTPase (PTP 1B) was first revealed, as

it had no similarity to known serine/threonine phosphatases (Tonks *et al.*, 1988). Since the original isolation of PTP 1B, more than 30 different PTPases have been isolated. A comparison of the PTPase and tyrosine kinase families reveals an interesting similarity. The general structures of the two tyrosine-directed enzymes parallel each other in that there both transmembrane or receptor-linked proteins as well as proteins that are wholly intracellular (Walton and Dixon, 1993).

The overexpression or activation of cellular PTKs, like the EGF receptor tyrosine kinase, leads to cell growth and proliferation (Reynolds *et al.*, 1981). There is evidence that in these systems PTPs have to act as negative regulators by maintaining the equilibrium of the cellular phosphotyrosine level. It is still possible that loss of PTPase activity may also be oncogenic because the human gene encoding a receptor-linked PTPase, PTPγ, is mapped to the chromosomal region 3p21, which is frequently deleted in renal cell carcinomas and lung carcinomas (LaForgia *et al.*, 1991).

HYPOTHESIS

Vitamin K_1 is the essential cofactor for the post-translational γ-carboxylation of a series of Glu residues in juxtaposition to the N-terminus of the vitamin K-dependent proteins (Shearer, 1992; Olson, 1984; Dowd *et al.*, 1995). These Gla residues facilitate the binding of these vitamin K-dependent proteins to cell phospholipid membrane in the presence of calcium (Furie and Furie, 1990). Vitamin K-dependent proteins are ligands for the RTKs that can regulate cellular proliferation and transformation (Varnum *et al.*, 1995). The vitamin K level in the mammalian fetus is tightly regulated by a maternal/fetal

placental gradient (Shearer *et al.*, 1982). This causes decrease in the levels of vitamin K-dependent ligands in growing fetus. On one hand the complete absence of vitamin K (in the presence of warfarin) inhibits embryo growth and development; on the other hand vitamin K levels above threshold increases the risk of cell transformation. We propose that vitamin K_1 may play a pivotal role in the regulation and stimulation of cellular growth and proliferation by controlling the γ-carboxylation of vitamin K-dependent ligands capable of binding to receptors with the capacity to promote growth and transformation. The low levels of vitamin K-dependent proteins in growing fetus may be necessary for normal embryonic development and may have some advantages to the fetus.

MATERIALS AND METHODS

I. CHICK EMBRYO MODEL AND VITAMIN K_1 ADMINISTRATION

Fertile eggs from Cornish hens were incubated at 37 °C and 85 % humidity and rotated hourly. At day 10 or 16 of incubation, all eggs were checked for fertilization and the air sac was marked for injection. A small hole was carefully made in the shell directly over the air sac with a needle. Varying amounts of vitamin K_1 (Sigma Chemical Co.) (in 10 μl acetone) alone or in combination with water-soluble warfarin were injected onto the inner membrane with a Hamilton syringe; noninjected eggs; eggs receiving 10 μl acetone alone served as controls and vehicle (acetone) controls, respectively. After 48 h incubation, the embryonic tissues (brain and liver) removed on day 12 and 18 were rinsed vigorously in ice with cold 1×PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) containing 1 mM sodium orthovanadate (Sigma Chemical Co.), and immediately frozen in liquid nitrogen till further protein extraction. The brain of the chicken embryo was removed by cutting longitudinally through the skull, and opening the cranial cavity.

II. PROTEIN EXTRACTION

Freshly frozen tissues were pounded in an iron tube to powder and homogenized in RIPA buffer (1 % Triton X-100, 1 % deoxycholate, 0.1 % SDS, 10 mM Tris-HCl, pH 7.6, 158 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, and 1 μg/ml aprotinin). After 30 min on ice, detergent-insoluble material was

pelleted by centrifugation at 14,000 rpm at 4 °C for 15 min. Protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL). Aliquots of detergent-soluble fraction were frozen with liquid nitrogen and stored at -80 °C.

III. SDS-PAGE AND IMMUNOBLOTTING

Triton soluble proteins (50 μg) were solubilized in SDS-sample buffer (final concentration was 1×SDS sample buffer, using 5×SDS sample buffer stock containing 62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2.3 % SDS, 100 mM dithiothreitol, 0.1 % bromophenol blue), boiled for 5 min, and protein separation by 7.5 % or 12 % SDS polyacrylamide gel electrophoresis (Laemmli, 1970). The proteins were then electrophoretically transferred onto nitrocellulose filters and the filters were blocked overnight at 4 °C with Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 3 % bovine serum albumin (BSA. Sigma Chemical Co.). Blots were incubated for at least 4 h with either primary antibody: anti-phosphotyrosine (anti-PY) antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), 1 μg/ml; anti-pp125^{FAK}, (Upstate Biotechnology Inc., Lake Placid, NY), 1 μg/ml; anti-pp60^{src} (Upstate Biotechnology Inc., Lake Placid, NY), 1 μg/ml; anti-paxillin (Transduction Laboratories, Lexington, KY), 1:5000; or anti-c-Eyk (gift of Dr. H. Hanafusa, The Rockefeller University, New York), 1:5000. After extensive washing in TBS containing 0.05 % Tween-20 (TBS-T), blots were incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG, Bio-Rad Laboratories,

Richmond, CA) 1:5000 in TBS-T for 1 h at room temperature. Bound antibodies were detected using enhanced chemiluminescence (Amersham, Oakville, Ontario, Canada). In some experiments, bound antibodies were removed by incubating the blot for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 100 mM 2-mercaptoethanol). The blots were then reprobed with specific antibodies against pp125^{FAK}, paxillin, pp60^{src} and c-Eyk. In a few experiments, the proteins were also visualized by silver staining according to the manufacturer's protocol (Bio-Rad Laboratories).

IV. IMMUNOPRECIPITATION

500 μg of detergent-soluble proteins from the brain and liver of day 12 and 18 embryos were pre-cleared by mixing with normal rabbit serum-coated protein A-Sepharose 4B (Pharmacia LKB Biotechnology AB Uppsala, Sweden) for 1 h at 4 °C. The clarified tissue extracts were added to protein A-Sepharose 4B beads previously incubated for 90 min with anti-pp125^{FAK}, anti-pp60^{src}, anti-paxillin or anti-c-Eyk antibodies. After 3 ~ 4 h incubation at 4 °C with gentle rocking, the beads were washed three times with cold lysis buffer containing 50 μM sodium orthovanadate, once with 0.5 M $LiCl_2$/0.1 M Tris-HCl (pH 7.4), and twice with 10 mM Tris-HCl (pH 7.4). The precipitated proteins recovered from the beads were subjected to immunoblotting studies and in vitro kinase assays.

V. PROTEIN-TYROSINE KINASE ASSAY

Aliquots (25 μg) of detergent-soluble protein in a total volume of 50 μl containing 50 μM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 μM sodium orthovanadate, 50 μM ATP, 2 μCi [γ-^{32}P] ATP (10 mCi/mL, Amersham) and 1 mg/mL poly (Glu/Tyr; 4:1), a synthetic tyrosine substrate (Sigma Chemical Co.), were incubated for 20 min at 30 °C. These reactions were stopped by the addition of 15 μl of boiling 5×SDS sample buffer. The phosphorylation of poly (Glu/Tyr; 4:1) was monitored after separation of proteins by 10 % SDS-PAGE followed by autoradiography. The blank reaction mixture, containing no peptide substrate or tissue protein, was processed identically, run in parallel lanes, and the counts from these lanes were subtracted from those containing both substrate and tissue protein (Maher and Pasquale, 1991).

VI. PROTEIN-TYROSINE PHOSPHATASE ASSAY

The reaction mixture (50 μl) containing 50 μg of detergent-soluble protein, 25 mM imidazole HCl (pH 7.2), 0.1 % β-mercaptoethanol, and 10 mM phosphotyrosine (Sigma Chemical Co.) was incubated for 10 min at 30 °C. After termination of the reaction by the addition of 50 μl of 10 mg/ml BSA and 150 μl of 25 % TCA, sample were vortexed, incubated for 10 min on ice, and centrifuged at 14,000×g for 5 min. The inorganic phosphate in the supernatant was assayed. Briefly, after the reaction was stopped and centrifuged, supernatant was removed to another tube and mixed with 1 mL of 10 % trichloroacetic acid. The tube was centrifuged at 14,000×g for 5 min. 0.5 mL of supernatant was removed into a 15-mL graduated centrifuge tube and adjusted the volume to 4 mL with distilled water. Then this tube was added 4 mL reagent C (mix 1 volume of 6

N sulfuric acid with 2 volumes of distilled water and 1 volume of 2.5 % ammonium molybdate, then add 1 volume of 10 % ascorbic acid and mix well, prepare fresh each day), capped with parafilm, and incubated tube in 37 °C water bath for 2 h. After incubation, the tube was allowed a few min to cool to room temperature, and the absorbance in Beckman DU spectrophotometer at wave-length 820 μm was read (Chen *et al.*, 1956). The blank reaction mixture, containing no tissue protein, was processed identically and the values were subtracted from those containing tissue protein. The presence of 200 μM sodium orthovanadate in the reaction mixtures inhibited > 95 % phosphatase activity measured by this assay.

VII. Src KINASE ASSAY

$pp60^{src}$ activity was assayed according to methods supplied by the manufacturer using synthetic peptides derived from $p34^{cdc2}$ (Upstate Biotechnology Inc.). Anti-$pp60^{src}$ immune complexes were incubated for 15 min at 30 °C with 50 μl of kinase reaction buffer containing 50 mM Tris-HCl (pH 7.0), 25 mM $MgCl_2$, 5 mM $MnCl_2$, 250 μM sodium orthovanadate, 100 μM [γ-^{32}P] ATP, and 300 μM substrate peptide. The reaction was terminated by the addition of 50 % acetic acid, and 25 μl aliquots of the reaction mixture were spotted onto strips of phosphocellulose filter paper. The strips were washed four times with excess 0.75 % phosphoric acid, once with acetone, and then dried. The dried strips were suspended in 5 ml of liquid scintillation fluid and counted for radioactivity.

VIII. MAP KINASE ACTIVITY ASSAY

500 μg of Triton soluble samples were pre-cleared for 2 h at 4 °C with 40 μl protein A-Sepharose 4B beads (Pharmacia LKB Biotechnology AB Uppsala, Sweden). The lysates were incubated with 5 μl (2.5 μg) rabbit anti-MAPK antibody (Upstate Biotechnology Inc.) for 2 h and 15 μl protein A-Sepharose 4B beads for another 2 h at 4 °C. Immune complexes were washed two times with Triton lysis buffer, two times with kinase buffer (KAB) containing 25 mM HEPES, pH 7.6; 20 mM $MgCl_2$; 20 mM glycerol phosphate; 1 mM sodium orthovanadate and 2 mM DTT. MAP kinase activity was assayed by resuspending the final beads in a total volume of 40 μl of KAB containing 0.25 mg/ml of Myelin Basic Protein (MBP. Sigma Chemical Co.), 1 μCi [γ-^{32}P] ATP, 50 μM cold ATP and incubated at 30 °C for 10 min. Assays were terminated by the addition of 15 μl 4×SDS sample buffer. These samples were immediately heated at 100 °C for 5 min and analyzed by electrophoresis on 12 % polyacrylamide gels. The gels were then fixed in 10 % acetic acid for 30 min, dried, and subjected to autoradiography. Phosphate incorporation was measured by excising substrate (MBP) bands from the gels and counting the radioactivity by liquid scintillation.

IX. IN VITRO PI 3-KINASE ASSSAYS

Tissue lysates (200 μg of protein) were incubated for 4 h at 4 °C with 4G10. Precipitates were washed two times with Triton lysis buffer containing 50 μM sodium orthovanadate and three times with 10 mM Tris-HCl, pH 7.4. PI 3-kinase activity was measured by adding 10 μg of sonicated L-phosphatidylinositol (PI. Sigma Chemical Co.) and 10 μCi of [γ-^{32}P] ATP in a volume of 40 μl of kinase reaction buffer containing 30

mM HEPES, pH 7.4; 30 mM $MgCl_2$; 50 μM cold ATP and 200 μM adenosine. Reactions were carried out for 15 min at room temperature and stopped by the addition of 100 μl of 1 N HCl and 200 μl of choroform:methanol (1:1, v/v). Lipids were separated on oxalate-treated TLC plates (EM Separations, A division of EM Industries, Inc. 480 Demorat Road. Gaibbstown, NJ, USA. 08027) using a solvent system of chloroform :methanol:water:28 % ammonia (45:35:7.5:2.5, v/v/v/v). TLC plates were exposed to X-ray film at -80 °C. Quantitation of radioactivity incorporated into lipids was performed by excising portions of the TLC plates following by liquid scintillation counting (Gold *et al.*, 1994).

RESULTS

I. ALTERATIONS IN LEVELS OR METABOLISM OF VITAMIN K_1-MODULATED PROTEIN-TYROSINE PHOSPHORYLATION IN CHICK EMBRYONIC TISSUES

1. Vitamin K_1 supplementation does not alter the overall expression of the proteins in chick embryonic tissues:

Silver-stained 7.5 % SDS-PAGE gel showing that administration of vitamin K_1 had no effect on the overall expression of the proteins in the brain and liver of day 12 chick embryos (Figure 6).

2. Effects of vitamin K_1 supplementation on tyrosine phosphorylation of proteins in chick embryos:

A major increase in tyrosine phosphorylation of proteins of apparent MW: 150-170, 120-130, 105-110, 67-70, and 55-60 kDa was observed at vitamin K_1 supplementation doses of 0.45 μg and 4.5 μg. Decreased tyrosine phosphorylation observed with the highest dose of vitamin K_1 (45 μg) may represent phylloquinone toxicity in the smaller chick embryo (Figure 7). A similar pattern of tyrosine phosphorylation was observed in the liver of vitamin K_1 supplemented chick embryos.

3. Effects of warfarin on tyrosine phosphorylation of proteins in chick embryos:

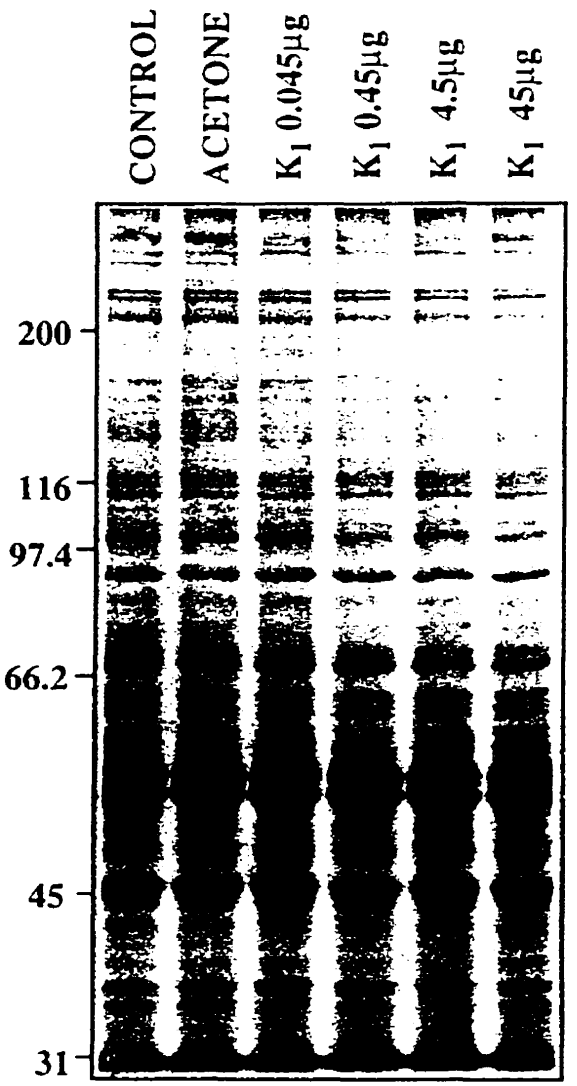

Figure 6. Silver-stained 7.5 % SDS-PAGE gel. The result showed no change in the expression of proteins in brain of day 12 embryo in the presence of vitamin K_1 (0.045 µg ~ 45 µg).

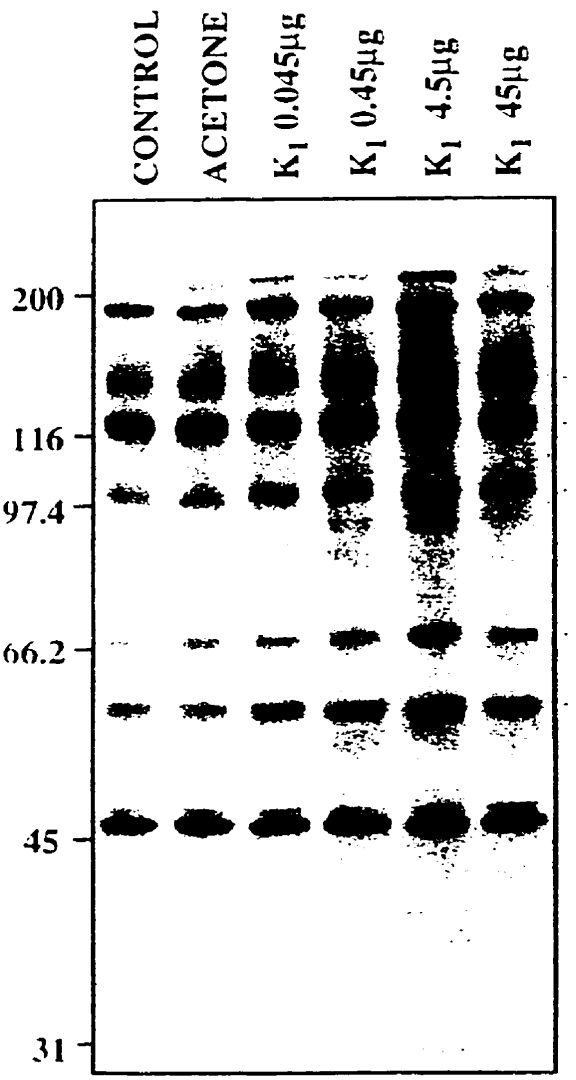

Figure 7. Vitamin K_1 induces protein-tyrosine phosphorylation during early embryogenesis. Eggs were injected with vitamin K_1 on day 10 and brain was removed 48 h later. Anti-phosphotyrosine immunoblot of detergent-soluble proteins from day 12 brain showing vitamin K_1-induced tyrosine phosphorylation. Equivalent results were obtained in five individual experiments.

Warfarin, an inhibitor of vitamin K_1 epoxide reductase, reduced tyrosine phosphorylation in a dose-dependent manner at a vitamin K_1 dose of 0.45 μg. At high warfarin doses, tyrosine phosphorylation of these proteins was downregulated to well below their basal level. The inhibitory effects of warfarin were not observed when the concentration of vitamin K_1 was increased to 4.5 μg, consistent with the provision of sufficient vitamin K_1 to by pass the metabolic block (Figure 8).

II. VITAMIN K_1-MEDIATED UPREGULATION OF PROTEIN-TYROSINE PHOSPHORYLATION AND PROTEIN-TYROSINE KINASE ACTIVITY DURING LATE EMBRYOGENESIS

1. Vitamin K_1 supplementation induces protein-tyrosine phosphorylation during late chick embryogenesis:

Studies have shown that the level of protein-tyrosine phosphorylation is highest in early developmental stages of the embryo, gradually decreases in late stages and almost undetectable in adult (Turner, 1991). When eggs were injected with vitamin K_1 on day 16 and brain tissue harvested 48 h later, a marked increase in tyrosine phosphorylation of proteins of MW: 150-170, 120-130, 105-110, 67-70, and 55-60 kDa was observed. The maximum effect on tyrosine phosphorylation in later embryo stage (18 day) was observed at vitamin K_1 concentration higher than that required during early embryo stage (day 12) (Figure 9).

2. Effects of vitamin K_1 supplementation on protein-tyrosine kinase activity in chick embryos:

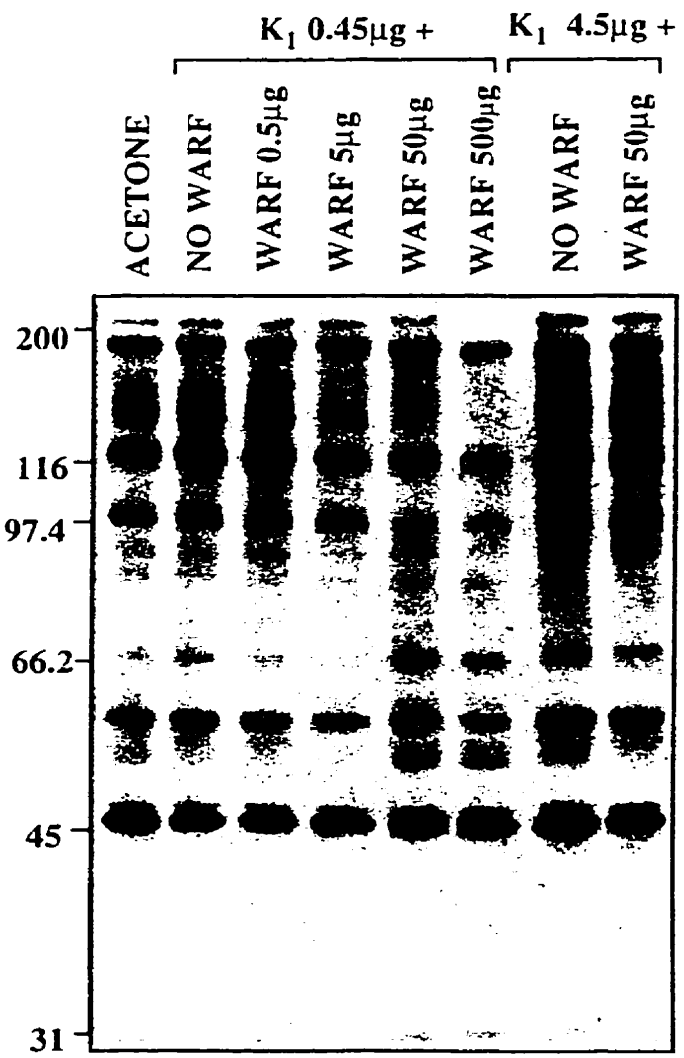

Figure 8. Warfarin reduced protein-tyrosine phosphorylation. Warfarin was injected together with varying doses of vitamin K_1. Anti-phosphotyrosine of detergent-soluble proteins from day 12 brain of chick embryos showing tyrosine phosphorylation. Equivalent results were obtained in five individual experiments.

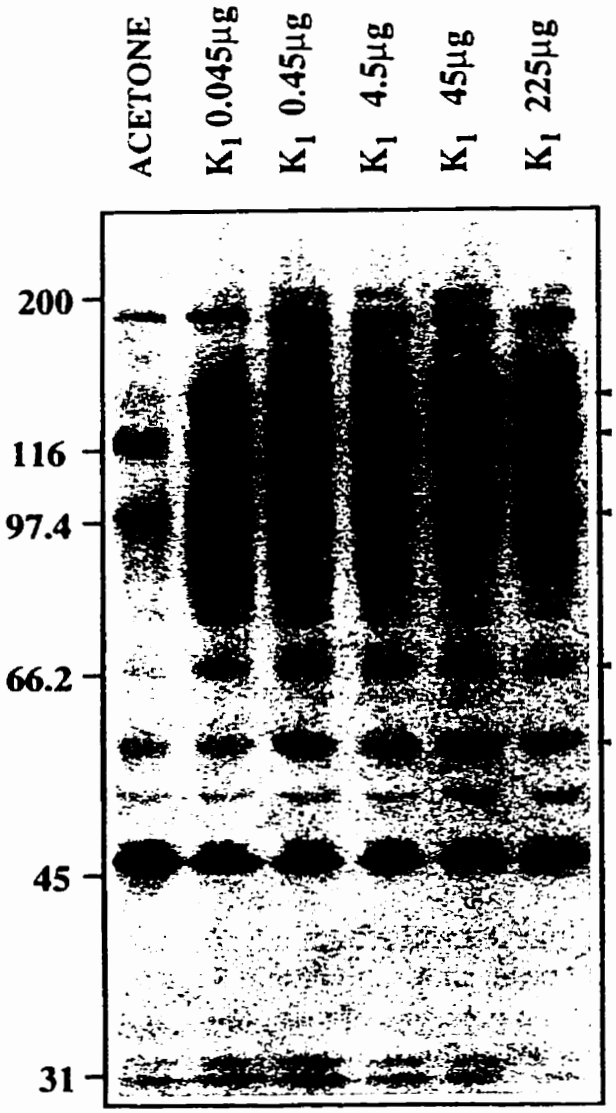

Figure 9. Anti-phosphotyrosine immunoblot of detergent-soluble proteins from day 18 chick brain. The result showed vitamin K_1-induced dose-dependent increase in tyrosine phosphorylation of several proteins. Equivalent results were obtained in five individual experiments.

Protein-tyrosine kinase activity was measured by the phosphorylation of synthetic random amino acid copolymer substrate. The data presented in Figure 10 indicates that PTK activity increases up to threefold with increasing doses of vitamin K_1 from 0.45 μg to 45 μg.

3. Effects of vitamin K_1 supplementation on protein-tyrosine phosphatase activity in chick embryos:

The protein-tyrosine phosphatase activity was measured by using phosphotyrosine (Sigma Chemical Co.) as substrate. After the reaction was stopped, the inorganic phosphate in supernatant was assayed as described in Materials and Methods. The data showed that the protein tyrosine phosphatase activity in brain of day 12 and day 18 chick embryos was not significant changed in presence of vitamin K_1.

III. INVOLVEMENT OF c-Eyk IN VITAMIN K_1-MEDIATED TYROSINE PHOSPHORYLATION CASCADE DURING CHICK EMBRYOGENESIS

Western blot analysis using anti-c-Eyk antibody identified the 105-110 kDa band that exhibited changes in its phosphorylation content in presence of vitamin K_1 and warfarin (Figure 7, 8, and 9) as c-Eyk. Anti-c-Eyk immunoprecipitates of day 12 brain of the control and the vitamin K_1 pretreated chick embryos were then analyzed to establish whether the observed changes are resident in post-translational tyrosine phosphorylation or due to increased synthesis of this protein. Immunoprecipitates were first analyzed with anti-phosphotyrosine immunoblotting, and subsequently with anti-c-Eyk antibody. While the c-Eyk immunoprecipitates from both the control and the vitamin K_1 pretreated chick

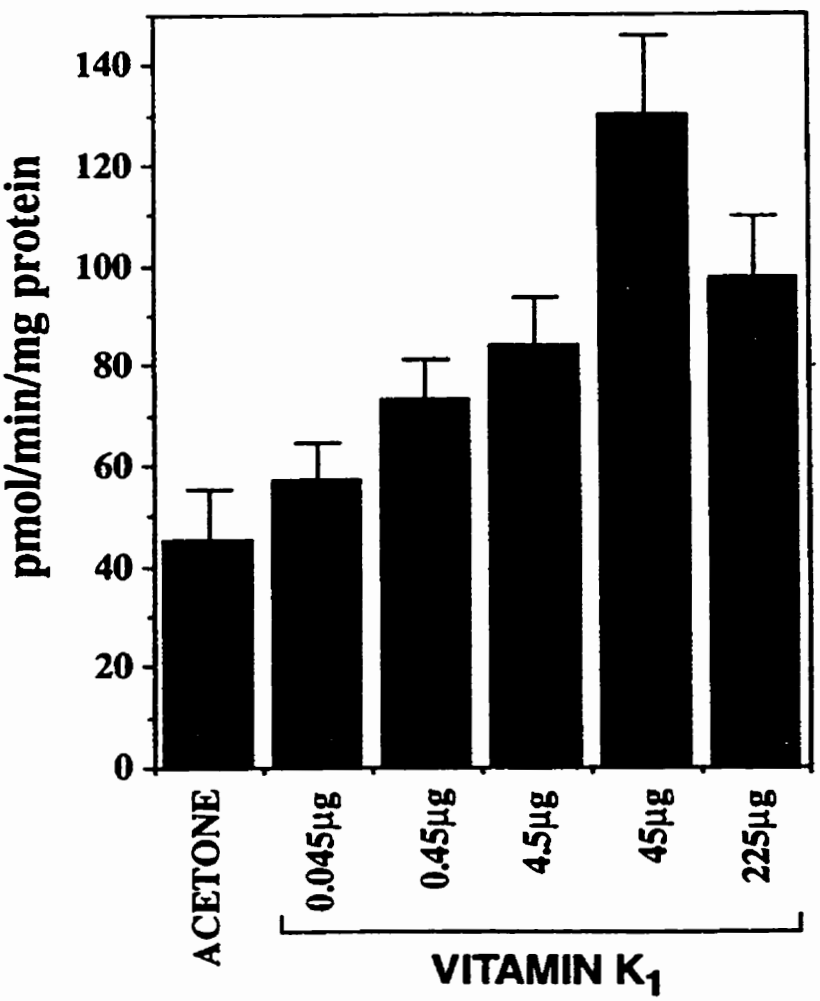

Figure 10. Vitamin K_1-induced protein-tyrosine kinase activity in day 18 chick embryonic brain. Data were plotted as the specific activity of the tyrosine kinase in pmol/min per mg protein. Results were the average of four determinations (mean±SE).

embryos contained equivalent amounts of c-Eyk protein, a marked increase in c-Eyk tyrosine phosphorylation was observed only in the vitamin K_1 pretreated chick embryos (Figure 11).

IV. FOCAL ADHESION KINASE ($pp125^{FAK}$), PAXILLIN, AND $pp60^{src}$ ARE COMPONENTS OF VITAMIN K_1-INDUCED SIGNALING PATHWAYS DURING EMBRYOGENESIS

To identify other components of the vitamin K_1-induced tyrosine phosphorylation cascade, we focused on proteins that exhibited modulation in the presence of vitamin K_1 (Figure 7 and 9). Reprobe of day 12 and 18 chick brain blots (Figure 7 and 9) with anti-$pp125^{FAK}$, anti-paxillin, and anti-$pp60^{src}$ antibodies confirmed the identity of the 120-130 kDa band as $pp125^{FAK}$, the 67-70 kDa band as paxillin, and the 55-60 kDa band as $pp60^{src}$. While anti-$pp125^{FAK}$ and anti-paxillin antibodies immunoprecipitated equal contents of $pp125^{FAK}$ and paxillin proteins from the brain and liver of both day 18 control and the vitamin K_1 pretreated chick embryos, only in the vitamin K_1 pretreated chick embryos were marked increases in tyrosine phosphorylation of these proteins (Figure 12).

Anti-$pp60^{src}$ immune-complexes isolated from chick embryos pretreated with vitamin K_1 exhibited up to a 2.5-fold increase in the phosphorylation of a synthetic peptide (KVRKIGEGTYGVVKK) derived from amino acids 6-20 of $p34^{cdc2}$ with Tyr-19 replaced by Lys (Figure 13).

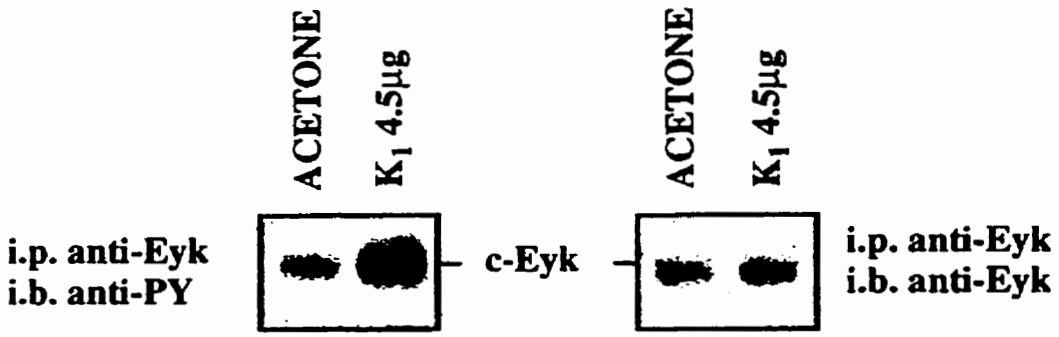

Figure 11. Tyrosine phosphorylation of c-Eyk in the brain of vitamin K_1 pretreated chick embryos. Increased tyrosine phosphorylation of c-Eyk in day 12 brain of vitamin K_1 pretreated chick embryos. Anti-c-Eyk immunoprecipitates (i.p.) from detergent-soluble lysates of day 12 brain were first immunoblotted (i.b.) with anti-phosphotyrosine antibody to measure the tyrosine phosphorylation of c-Eyk (left), then stripping and reprobed with anti-c-Eyk antibody for protein expression (right). Equivalent results were obtained in three individual experiments.

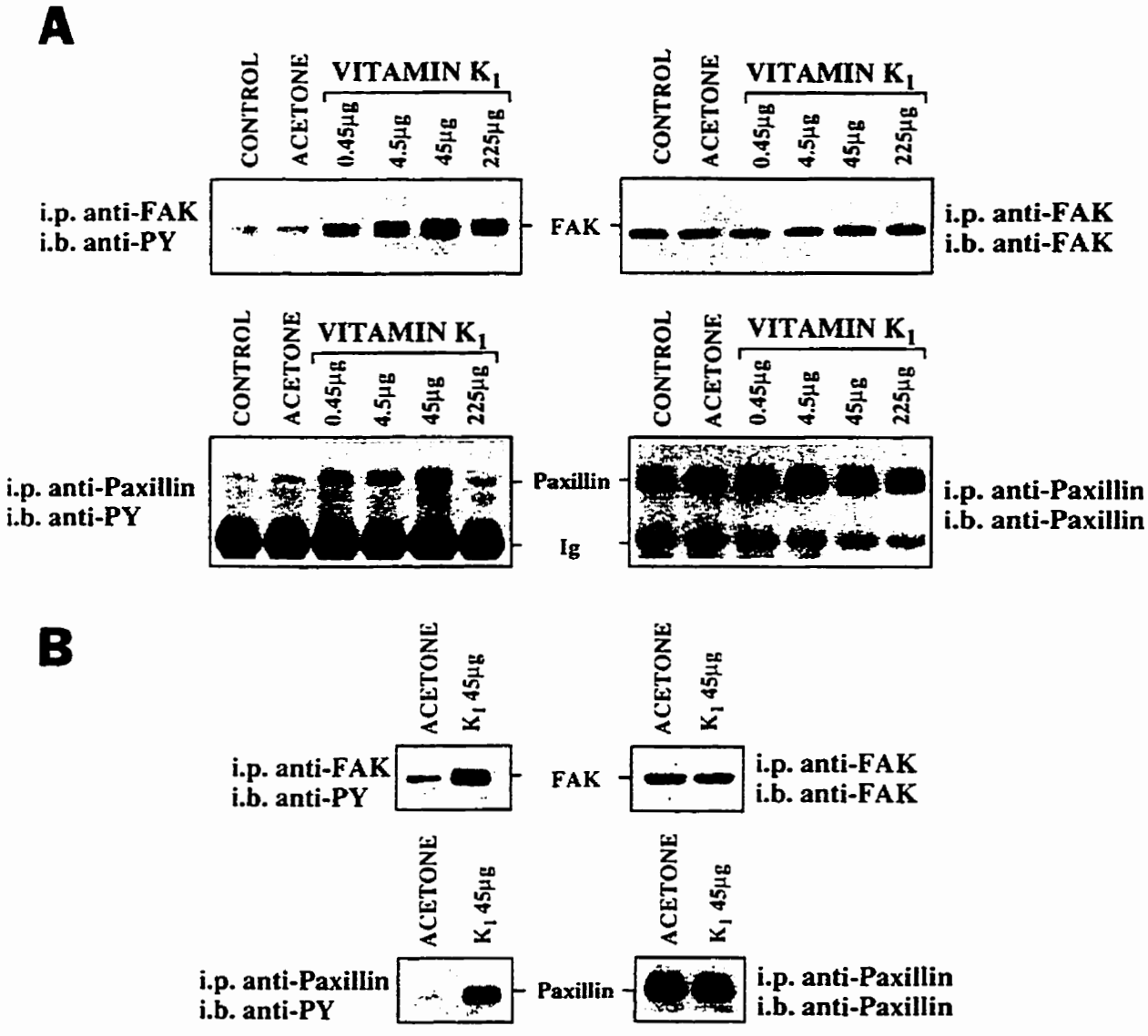

Figure 12. Identification of pp125^{FAK} and paxillin as tyrosyl proteins modulated by vitamin K_1 in day 18 brain and liver of chick embryos. pp125^{FAK} and paxillin were immunoprecipitated (i.p.) from detergent-soluble lysates of brain (A) and liver (B) of day 18 embryos. Blots were analyzed by anti-phosphotyrosine immunoblotting (i.b.) to measure vitamin K_1-induced changes in tyrosine phosphorylation. The blots were stripped and reprobed with anti-pp125^{FAK} or paxillin antibodies for protein expression. Equivalent results were obtained in three individual experiments.

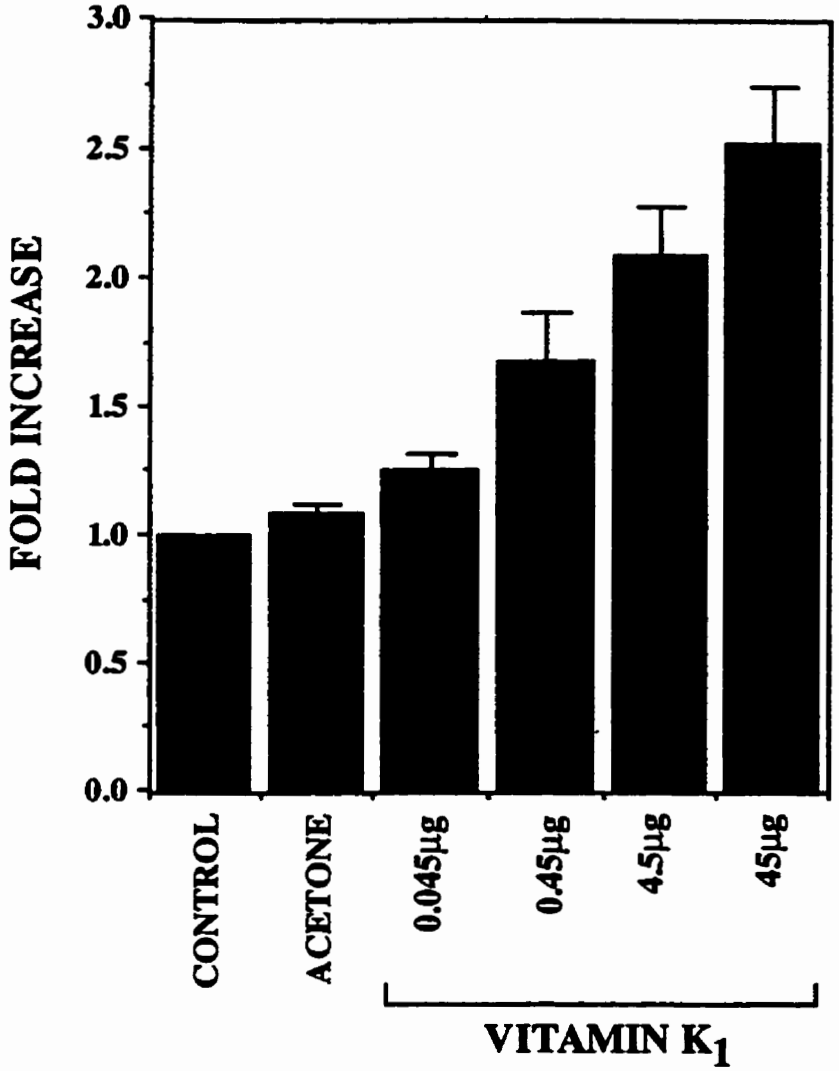

Figure 13. pp60^{src} activity in chick embryos. Anti-pp60^{src} immune complexes isolated from day 18 brain of chick embryos supplemented with vitamin K_1 exhibited increased phosphorylation of p34^{cdc2} peptides.

DISCUSSION

Protein-tyrosine phosphorylation plays an important role in precise regulation of cell division, differentiation, and migration required for normal embryogenesis (Sprenger *et al.*, 1989; Basler *et al.*, 1988; Aroian *et al.*, 1990; Raff *et al.*, 1988; Barres *et al.*, 1992). Recently, protein S, a vitamin K-dependent coagulation inhibitor (Dahlback, 1991), and its relative Gas6 (vitamin K-dependent protein), a protein encoded by the growth-arrest specific gene 6 (Manfioletti *et al.*, 1993), have been identified as classical ligands for a unique family of RTKs (Tyro 3, 7, 12) (Stitt *et al.*, 1995; Godowski *et al.*, 1995; Ohashi *et al.*, 1995). The involvement of vitamin K metabolism and function in two well characterized human birth defects, warfarin embryopathy (Hall *et al.*, 1980) and vitamin K epoxide reductase deficiency (Pauli *et al.*, 1987) is also reported. These studies suggest the requirement of a vitamin K-dependent pathway(s) in orderly embryogenesis. The results presented here confirm the existence of a vitamin K_1-sensitive tyrosine phosphorylation cascade during embryogenesis. This cascade involves key intracellular proteins, including $pp125^{FAK}$, paxillin, and $pp60^{SRC}$. Supplementation of vitamin K_1 both at an early stage (day 10) and late stage (day 16) significantly increased tyrosine phosphorylation of these proteins (Figure 7, 9, 11, 12). These studies demonstrate that vitamin K_1-dependent signals may play a pivotal role in chick embryogenesis. Based on studies using warfarin, the effects of vitamin K_1 during embryogenesis appear to be mediated by a mechanism involving the γ-carboxylation of vitamin K-dependent proteins.

We have not yet ruled out the possibility of other mechanism(s) that may work independent of vitamin K_1.

The overall level of tyrosine phosphorylation is high in chick embryonic tissue during the early stages of development, decreases significantly during late embryogenesis, and is low or undetectable in the same tissues of the adult (Maher *et al.*, 1988). We used the chick embryo as a model for embryogenesis because it is an easily accessible *in vivo* system to study cell signaling pathways during development. Well defined organ development is present by day 10 of chick embryo. In our studies, vitamin K_1 supplementation, both at an early stage (day 12) and at a late stage (day 18) of chick embryos, significantly increased the tyrosine phosphorylation of several proteins in brain (Figure 7, 9) and liver (Figure 12 B). These tyrosine phosphorylated proteins, modulated in the presence of vitamin K_1, are similar to those previously shown to exhibit temporal changes in their levels of tyrosine phosphorylation during normal chick embryonic development (Maher *et al.*, 1988). The effects of vitamin K_1, on protein tyrosine phosphorylation were due neither to an effect on the overall expression of these proteins (Figure 6), nor to an effect on protein-tyrosine phosphatase activity. In addition, vitamin K_1 supplementation caused an increase, up to threefold, in protein-tyrosine kinase activity in day 18 chick embryonic brain (Figure 10). The effects of vitamin K_1 on tyrosine phosphorylation in chick embryos were observed with doses of vitamin K_1 at or well below the usual prophylactic dose given to the full term human neonate. Warfarin, an inhibitor of vitamin K_1 epoxide reductase, that interrupts the recycling of vitamin K_1 from

the epoxide to the hydroquinone form, inhibited the effects of low dose vitamin K_1 (0.45 μg) on tyrosine phosphorylation in the brain of day 12 chick embryos; no inhibition was observed at higher vitamin K_1 (4.5 μg) level (Figure 8). These studies indicate that higher vitamin K_1 levels are sufficient to bypass the metabolic block. The demonstration of the inhibitory effects of warfarin on protein tyrosine phosphorylation during embryonic development provides a possible explanation for the fetal toxicity of this drug.

c-Eyk, a 106 kDa chicken counterpart of the Tyro 12 family of RTKs, exhibits a broad spatial and temporal expression during embryonic development (Jia and Hanafusa, 1994). The 105-110 kDa band, which exhibited major changes in tyrosine phosphorylation in chicken embryos with vitamin K_1 and warfarin supplementation (Figure 7, 8 and 9), was identified as c-Eyk by western blot analysis. Analysis of c-Eyk immunoprecipitates from the detergent-soluble lysates of day 12 brain of chicken embryos demonstrated that vitamin K_1 induced a major increase in its tyrosine phosphorylation but did not cause any change in the expression of c-Eyk protein (Figure 11). Although protein S and its relative Gas6, have been identified as classical ligands for the Tyro3, 7 & 12 family of RTKs (Stitt *et al.*, 1995; Godowski *et al.*, 1995; Ohashi *et al.*, 1995), the ligand of c-Eyk has still not been identified. Although our studies suggest the involvement of c-Eyk in vitamin K_1-induced tyrosine phosphorylation cascade, other receptor systems mediating may also be the effects of vitamin K_1 during embryonic development.

A link between the extracellular matrix (ECM) and the actin cytoskeleton is made at focal adhesion sites (Burridge *et al.*, 1988). These focal adhesions not only provide an anchor to which the cytoskeleton can apply stress, but are also involved in transducing signals between extracellular and intracellular milieu (Juliano and Haskill, 1993; Luna and Hitt, 1992). Protein expression and tyrosine phosphorylation of a cytoplasmic pp125^{FAK} and its potential substrate paxillin are under developmental control (Turner *et al.*, 1993; Turner, 1991). Our results demonstrate that vitamin K_1 supplementation induces tyrosine phosphorylation of pp125^{FAK} and paxillin in brain and liver of the day 18 chick embryo without modifying the expression of these proteins (Figure 12). It is known that autophosphorylation of pp125^{FAK} at tyrosine Tyr-397 generates an SH-2 mediated interaction with a member of the Src family (Schaller *et al.*, 1994). This interaction enzymatically activates the Src family kinase which, in turn, phosphorylates Tyr-407, Tyr-576, and Tyr-577 of pp125^{FAK} to fully activate this kinase (Calalb *et al.*, 1995). Consistent with these observations, the 55-60 kDa band that exhibited increased tyrosine phosphorylation in the presence of vitamin K_1 (Figure 7, 9) was identified as pp60^{src} by Western blot analysis using anti-pp60^{src} antibody. Furthermore, anti-pp60^{src} immune complexes isolated from chick embryos pretreated with vitamin K_1 showed up to a 2.5-fold increase in the phosphorylation of a synthetic peptide (Figure 13). The concomitant increase in the tyrosine phosphorylation of pp125^{FAK} as well as paxillin, an *in vivo* substrate of both pp125^{FAK} and pp60^{src} (Turner, 1994), is consistent with the propagation of growth regulatory signals in a vitamin K_1-induced cascade (Figure 12 A, B), and

suggests that alterations in the levels of vitamin K_1 during embryogmesis may result in dysregulation of cell-cell or cell-matrix adhesion and other growth regulatory pathways.

It has been reported that warfarin used in conjunction with chemotherapy increase the median survival time for patients with small cell carcinoma of the lung (Zacharski *et al.*, 1981) and with metastatic adenocarcinoma of the colon (Chlebowski *et al.*, 1982). *axl* has also shown to overexpress in metastatic colon cancer (Craven *et al.*, 1995). Recently, vitamin K_1 was shown to inhibit the activity of lapachol, a novel anticancer agent and a vitamin K_1 antagonist. Lapachol may target vitaminK_1-dependent reactions, including the Gas-Axl interaction and in turn might block the transduction of signals that stimulate proliferation in tumors where transforming RTKs, like *axl*, are expressed (Dinnen and Ebisuzaki, 1997). These studies suggest that activation in vitamin K_1 level may regulate the activity of RTKs by controlling the receptor-ligand interactions.

In most western countries vitamin K_1 is now administered orally or intramuscularly to prevent hemorrhagic disease due to low levels of the vitamin K-dependent coagulation factors at the time of birth (Kries *et al.*, 1988). Cord blood concentrations of vitamin K_1 in newborns ranged from 4 ~ 45 pg/mL, while their mothers had values between 144 ~ 2420 pg/mL, with a median maternal to cord blood of newborns ratio of about 30:1. These low vitamin K_1 concentrations in cord blood of newborns seem to reflect, at least in part, the very limited placental transfer of the vitamin K_1 (Shearer *et al.*, 1982; Shearer *et al.*, 1983). The administration of vitamin K_1 to the newborn results in a 3 to 4 log increase in

hepatic vitamin K_1 concentration compared with newborn who had not received vitamin K_1 (Guillaumont *et al.*, 1993). The inhibition of protein-tyrosine phosphorylation by warfarin is consistent with its known toxicity to the human fetus (Hall *et al.*, 1980), as warfarin crosses the placenta and results in fetal death or skeletal anomalies similar to those described in congenital vitamin K epoxide reductase deficiency (Pauil *et al.*, 1987). The present data explain, at least in part, why the levels of vitamin K and vitamin K-dependent ligands in the fetus and embryo are tightly controlled and maintained at low levels: their complete absence (in the presence of warfarin) inhibiting cell growth and development; their increase above threshold levels increasing the risk of cell transformation. The vitamin K-dependent receptor-ligand system appears to be involved in growth regulatory pathway during embryogenesis.

In summary, we have demonstrated that vitamin K_1 is an important element in embryonic development, and the existence of a vitamin K_1-dependent protein-tyrosine phosphorylation cascade involving c-Eyk, a member of the Tyro 12 family, and key intracellular proteins, including $pp125^{FAK}$, paxillin, and $pp60^{src}$. This cascade is sensitive to alteration in levels or metabolism of vitamin K_1. These findings provide a major clue as to why, in the mammalian fetus, the vitamin K-dependent proteins are maintained in an undercarboxylated state, even to the point of placing the newborn at hemorrhagic risk. These studies therefore suggest that precise regulation of vitamin K-dependent regulatory pathways may be critical for orderly embryogenesis.

SIGNIFICANCE

The total body pool of vitamin K is tightly controlled both in the adult and in the fetus. In the mammalian fetus, vitamin K_1 is regulated by a maternal/fetal placental gradient; the median vitamin K_1 concentration in human cord plasma is 16 pg/mL as compared with a maternal median plasma level of 470 pg/mL. This lower vitamin K_1 concentration in the fetus and newborn is reflected in reduced γ-carboxylation of coagulation factors II, VII, IX, and X, Protein C and S, and bone protein matrix-Gla protein and osteocalcin. Although delayed osteocalcin production without rigid skeletal formation may be of benefit to the fetus, the low levels of the coagulation factors with the attendant hemorrhagic risk are difficult to explain.

In recent years, supplementation of the newborn with vitamin K at birth and the resulting high levels have became a focal issue following the publication of a paper by Golding *et al* in 1992. This epidemiological study reported an increased incidence of childhood cancer in those children from the Bristol area of Great Britain given intramuscular vitamin K at birth. This association was not confirmed by subsequent studies in Sweden (Ekelund *et al.*, 1993) and the USA (Klebanoff *et al.*, 1993). Although the administration of 0.5 ~ 2.0 mg of vitamin K_1 immediately post-delivery is routine in North America, the basis of this regimen has been questioned in Europe (Kunzer *et al.*, 1983). The controversy arises in part from the fact that we are intervening in a natural phenomenon. The question as to why vitamin K levels in the fetus are maintained below

that required to provide full γ-carboxylation of the vitamin K-dependent proteins and why, even in the adult, no major vitamin K tissue stores exist remains unanswered.

Using a chick model of embryogenesis, the present study demonstrate the existence of a vitamin K_1-dependent protein-tyrosine phosphorylation cascade involving c-Eyk and key intracellular proteins, including $pp125^{FAK}$, paxillin, and $pp60^{src}$. These cascade is sensitive to alteration in levels or metabolism of vitamin K_1. These finding provide a major clue that low vitamin K and undercarboxylated state of vitamin K-dependent proteins may be benefit to the fetus — even to the point of placing the newborn at hemorrhagic risk. This explains, at least in part, why the levels of vitamin K and vitamin K-dependent ligands in the fetus and embryos are tightly controlled and maintained at low levels: their complete absence (in the presence of warfarin) inhibiting cell growth and development; their increase above threshold levels increasing the risk of cell transformation. These studies also suggest that precise regulation of vitamin K-dependent regulatory pathway may be critical for orderly embryogenesis.

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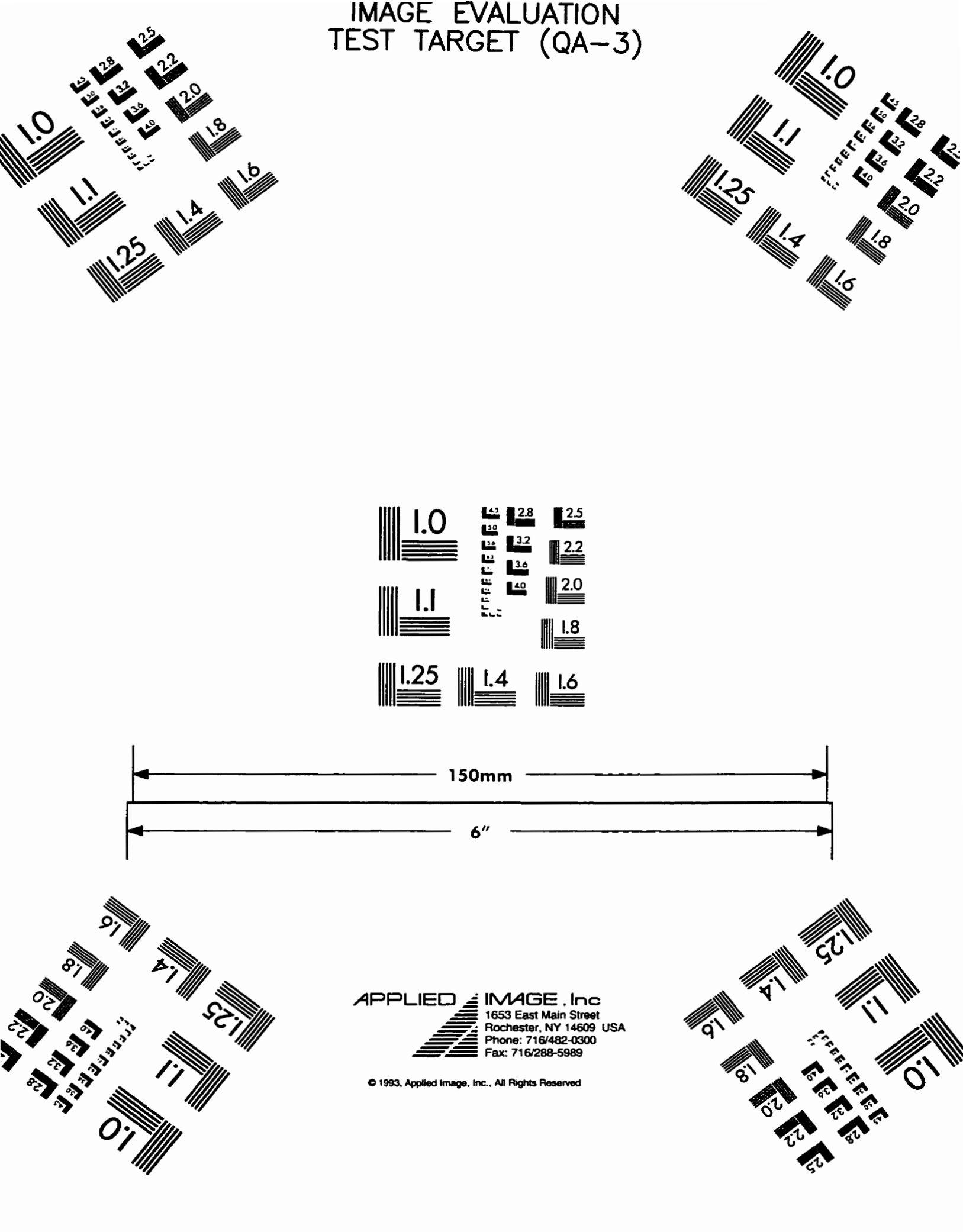
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