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Research Article

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A Novel Role for Vitamin K_1 in a Tyrosine Phosphorylation Cascade during Chick Embryogenesis

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Abstract

The development of the embryo is dependent upon a highly coordinated repertoire of cell division, differentiation, and migration. Protein-tyrosine phosphorylation plays a pivotal role in the regulation of these processes. Vitamin K-dependent γ-carboxylated proteins have been identified as ligands for a unique family (Tyro 3 and 7) of receptor tyrosine kinases (RTKs) with transforming ability. The involvement of vitamin K metabolism and function in two well characterized birth defects, warfarin embryopathy and vitamin K epoxide reductase deficiency, suggests that developmental signals from K-dependent pathways may be required for normal embryogenesis. Using a chick embryogenesis model, we now demonstrate 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 focal adhesion kinase ($pp125^{FAK}$), 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 (and human) fetus, the K-dependent proteins are maintained in an undercarboxylated state, even to the point of placing the newborn at hemorrhagic risk. The precise regulation of vitamin K_1-dependent regulatory pathways would appear to be critical for orderly embryogenesis. (*J. Clin. Invest.* 1997. 99: 602–607.) Key words: K-dependent proteins • receptor tyrosine kinases • warfarin • focal adhesion kinase • paxillin

Introduction

Vitamin K_1 (2 methyl-3-phytyl-1, 4 napthoquinone) is an essential cofactor for the carboxylase involved in the posttranslational γ-carboxylation of a series of glutamic acid residues in juxtaposition to the NH_2 terminus of the vitamin K-dependent proteins (1–3). These γ-carboxyglutamic acid (Gla)[1] residues facilitate the binding of vitamin K-dependent proteins to membrane phospholipids in the presence of calcium (4, 5). The vitamin K_1 level in the mammalian fetus is tightly regulated by a maternal/fetal placental gradient (6); 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, Proteins C and S, and bone protein matrix-Gla protein and osteocalcin (7). 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 is difficult to explain.

The identification of several receptor and nonreceptor kinases in embryonic tissues (8, 9), accompanied by major temporal changes in the levels of protein–tyrosine phosphorylation (10) and tyrosine kinase activity (11) during embryonic development, suggests that such posttranslational modification of target proteins is key to the regulation of orderly embryogenesis. The extensive sequence similarity shared by tyrosine kinase domains has helped in homology-based cloning of a unique group of receptor tyrosine kinases (RTKs), Tyro 3 (alternatively called Sky, Rse, Brt, or Tif), Tyro 7 (alternatively called Axl, UFO, or Ark), and Tyro 12 (alternatively called c-Eyk or c-mer) (12). The extracellular domain of theTyro 3, 7, and 12 family of RTKs consists of a combination of fibronectin type III and immunoglobulin motifs common to extracellular matrix proteins, neural cell adhesion molecules, and cell surface receptors with tyrosine kinase or phosphatase activities (13, 14). It is believed that these RTKs may be bifunctional, acting both as cell adhesion proteins and as components of signal transduction. Recently, Protein S, a vitamin K-dependent coagulation inhibitor (15), and its relative Gas6, a protein encoded by the growth arrest specific gene (16), have been identified, respectively, as classical ligands for the Tyro 3 and Tyro 7 family of RTKs (17–22). The role of this novel vitamin K-dependent receptor-ligand system in cellular processes is not clear but studies showing transforming activity of Axl in NIH 3T3 cells (23, 24), and the mitogenic potential of Protein S in smooth muscle cells (25) suggest a role in growth regulation.

The chick embryo has been used as a model system to study the signaling pathways operative during embryonic development. Since c-Eyk, a chicken counterpart of the Tyro 12 family of RTKs, exhibits a broad spatial and temporal expression during development (26), we sought to explore a possible vitamin K-dependent regulatory pathway(s) in a chick model of embryogenesis. We report the existence of a vitamin K_1-dependent protein-tyrosine phosphorylation cascade involving c-Eyk and key intracellular proteins, including focal adhesion kinase ($pp125^{FAK}$), paxillin, and $pp60^{src}$, which are all integral to chick embryogenesis.

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1. *Abbreviations used in this paper:* anti-PY, anti-phosphotyrosine; Gla, γ-carboxyglutamic acid; $pp125^{FAK}$, focal adhesion kinase; RTKs, receptor tyrosine kinases.

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Methods

Animal model and drug administration. Fertile eggs from Cornish hens were incubated at 37°C and 85% humidity and rotated hourly. At days 10 or 16, a small hole was made in the shell directly over the air sac and varying amounts of vitamin K_1 (in 10 μl acetone) alone or in combination with water soluble warfarin were injected onto the inner membrane with a Hamilton syringe; noninjected eggs and eggs receiving 10 μl acetone alone served as controls and vehicle (acetone) controls, respectively. After 48 h incubation, the embryonic tissues removed on days 12 and 18 were rinsed vigorously in ice cold phosphate-buffered saline with 1mM sodium orthovanadate and used for protein extraction.

Protein extraction. Freshly harvested tissues were homogenized in lysis buffer containing 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 removed by centrifugation at 14,000 rpm at 4°C for 15 min. Aliquots (5–10 μl) of supernatant were used for protein determination by a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL).

Immunoprecipitation. 500 μg of detergent soluble protein from the brain and liver of day 12 or day 18 embryos were pre-cleared by mixing with normal rabbit serum-coated protein A-Sepharose 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) for 1 h in the cold. The clarified tissue extracts were added to protein A–Sepharose beads previously incubated for 90 min with anti-$pp125^{FAK}$ (Upstate Biotechnology Inc., Lake Placid, NY), anti-$pp60^{Src}$ (Upstate Biotechnology Inc.), anti-paxillin (Transduction Laboratories, Lexington, KY) or anti-Eyk antibodies (gift of Dr. H. Hanafusa, The Rockefeller University, New York). 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–HCI (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.

SDS-PAGE and immunoblotting. 50 μg protein samples of tissue extracts or immunoprecipitates were solubilized in SDS-sample buffer (62.5mM Tris, 10% glycerol, 2.3% SDS, 100 mM dithiothreitol, 0.1% bromophenol blue, pH 6.8), boiled for 5 min, and electrophoresed on 7.5% SDS–polyacrylamide gel electrophoresis (27). The proteins were then electrophoretically transferred onto nitrocellulose, filters blocked overnight in the cold with Tris-buffered saline containing 3% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Blots were incubated for at least 4 h with primary antibody (an anti-phosphotyrosine (anti-PY) antibody 4G10 (Upstate Biotechnology Inc.), 1 μg/ml; anti-$pp125^{FAK}$, 1 μg/ml; anti-$pp60^{Src}$, 1 μg/ml; anti-paxillin, 1:5,000; or anti-Eyk, 1:5,000. After extensive washing in Tris-buffered saline containing 0.05% Tween-20, blots were incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit, 1:5,000 in TBST) (Bio-Rad Laboratories, Richmond, CA) for 1 h at room temperature. Bound antibodies were detected using enhanced chemiluminescence (Amersham, Oakville, Ontario, Canada). Bound antibodies were removed by incubating the blot for 30 min at 50°C in 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).

Protein tyrosine kinase assay. Protein tyrosine kinase assays were conducted according to the method described by Maher in reference 11. Briefly, 25-μg aliquots 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 kinase substrate (Sigma Chemical Co.), were incubated for 20 min at 30°C. The reactions were stopped by the addition of 15 μl of boiling 5 × SDS sample buffer. The phosphorylation of

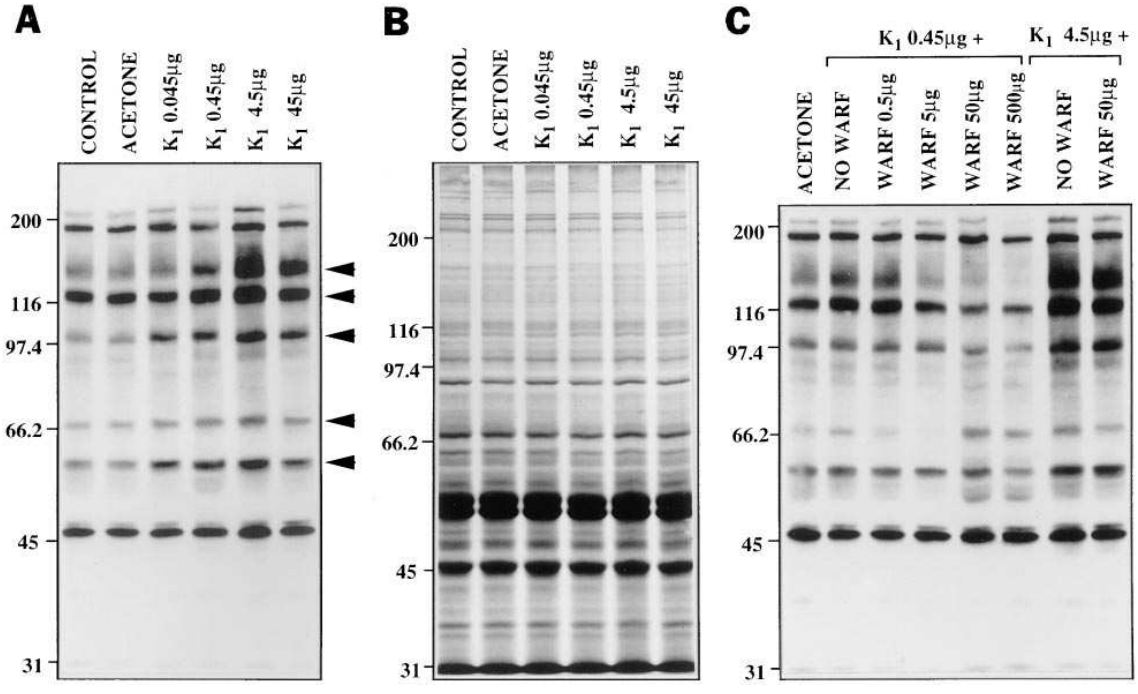

Figure 1. Vitamin K_1 induces protein tyrosine phosphorylation during early embryogenesis. Eggs were injected with vitamin K_1 on day 10 and brain tissue was obtained 48 h later. (*A*) Anti-PY immunoblot of detergent soluble proteins from day 12 brain showing vitamin K_1-induced tyrosine phosphorylation. (*B*) Silver-stained gel showing no change in the level of day 12 brain proteins in the presence of vitamin K_1. (*C*) As *A*, except that warfarin was injected together with varying doses of vitamin K_1. Equivalent results were obtained in five individual experiments. The positions of tyrosine phosphorylated proteins exhibiting changes, and $M_r \times 10^{-3}$ are indicated.

poly (Glu/Tyr; 4:1) was monitored after separation of proteins by SDS-PAGE (10%) 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.

Protein tyrosine phosphatase assay. The assay for protein tyrosine phosphatase activity used phosphotyrosine (Sigma Chemical Co.) as substrate. The reaction mixture (50 μl) containing 50 μg of detergent soluble proteins, 25 mM imidazole HCl (pH 7.2), 0.1% β-mercaptoethanol, and 10 mM phosphotyrosine was incubated for 10 min at 30°C. After termination of the reaction by the addition of 50 μl of 10 mg/ml bovine serum albumin and 150 μl of 25% TCA, samples 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 as described in reference 28. 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.

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-32P]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 was spotted onto a phosphocellulose filter paper strip. The strips were washed four times with excess 0.75% phosphoric acid, once with acetone, and then dried. The dried strips suspended in 5 ml of liquid scintillation fluid were counted for radioactivity.

Results

Alterations in levels or metabolism of vitamin K_1 modulate protein tyrosine phosphorylation in embryonic tissues. Although administration of vitamin K_1 had no effect on the overall expression of the proteins in the brain of day 12 embryos (Fig. 1 *B*), a major increase in tyrosine phosphorylation of proteins of apparent M_r 150–170, 120–130, 105–110, 67–70, and 55–60 kD was observed at vitamin K_1 doses of 0.45 μg and 4.5 μg per embryo (Fig. 1 *A*). The vitamin K_1-dependent modulation of tyrosine phosphorylation is not exclusive to brain—a similar pattern was observed in the liver of these embryos (data not shown). Decreased tyrosine phosphorylation observed with the highest dose of vitamin K_1 (45 μg) may represent phylloquinone toxicity in the smaller embryo. Warfarin, an inhibitor of K_1 epoxide reductase, reduced tyrosine phosphorylation in a dose-dependent manner at a K_1 dose of 0.45 μg. The inhibitory effects of warfarin did not occur 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. At high warfarin doses, tyrosine phosphorylation of many proteins was downregulated to well below their basal level (Fig. 1 *C*).

Vitamin K_1 upregulates protein tyrosine phosphorylation and protein tyrosine kinase activity in late embryogenesis. As it has been demonstrated previously (10) that tyrosine phosphorylation decreases towards the time of hatch (day 21), we examined the vitamin K_1 effect in older embryos. Consistent with previous reports, control values for tyrosine phosphorylation

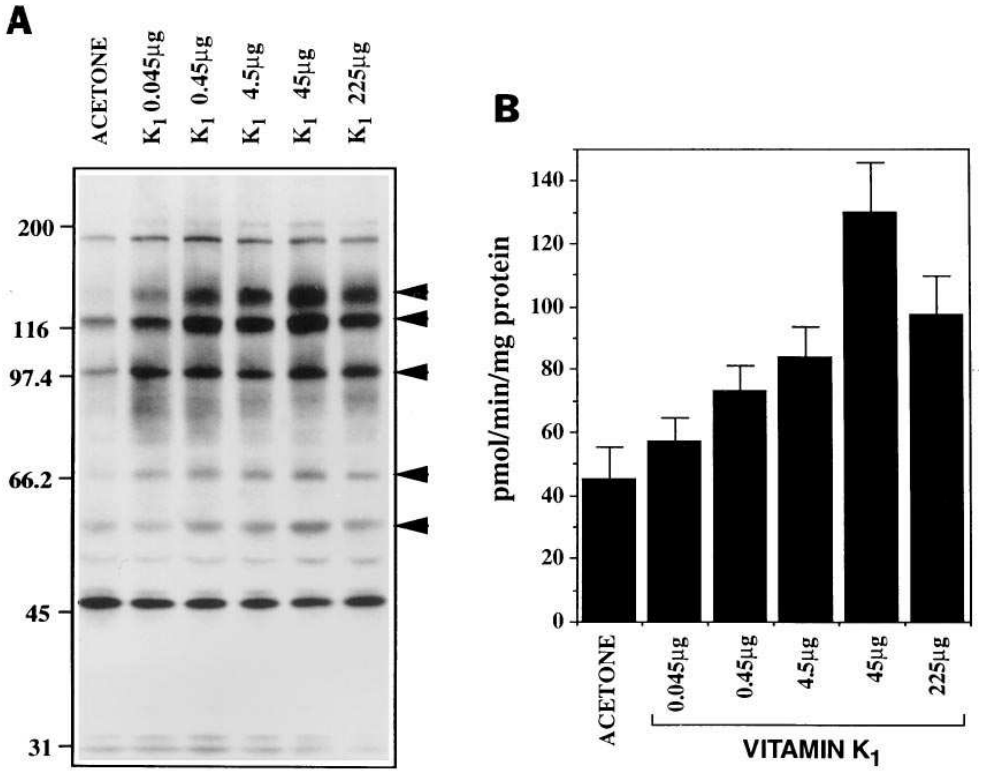

Figure 2. Vitamin K_1 upregulates the lower levels of protein tyrosine phosphorylation and tyrosine kinase activity in late embryogenesis. (*A*) Anti-PY immunoblot of detergent soluble proteins from day 18 brain showing vitamin K_1-induced dose-dependent increase in tyrosine phosphorylation of several proteins. Eggs were injected with vitamin K_1 on day 16 and brain tissue obtained 48 h later was homogenized in RIPA lysis buffer as described in Methods. Proteins (50 μg) separated by SDS-PAGE (7.5%) were transferred to nitrocellulose and blots probed with an anti-PY antibody. Equivalent results were obtained in five individual experiments. The positions of tyrosine phosphorylated proteins exhibiting changes, and $M_r \times 10^{-3}$ are indicated. (*B*) Vitamin K_1-induced protein tyrosine kinase activity in day 18 embryonic brain. The protein-tyrosine kinase activity was measured by the phosphorylation of synthetic random amino acid copolymer substrate as described in reference 11. Data are plotted as the specific activity of the tyrosine kinases in pmol/min per mg protein. Results are the average of four determinations (mean±SE).

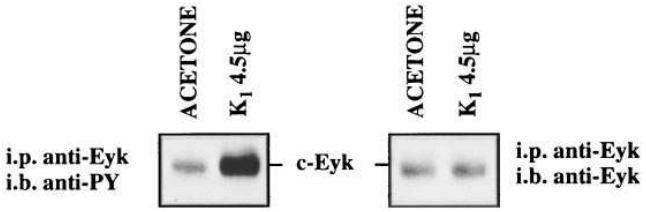

Figure 3. Increased tyrosine phosphorylation of c-Eyk in the brain of vitamin K_1-pretreated day 12 embryos. After injection of vitamin K_1 on day 10, brain tissue obtained on day 12 was homogenized in RIPA lysis buffer and c-Eyk immunoprecipitated as described. Anti-Eyk immunoprecipitates (i.p.) from detergent soluble lysates of brain of day 12 embryos were immunoblotted (i.b.) with anti-PY antibody to monitor the tyrosine phosphorylation of c-Eyk, and reprobed with anti-Eyk antibody for protein expression. Equivalent results were obtained in three individual experiments.

on day 18 were significantly lower than those on day 12 (Figs. 2 *A* and 1 *A*); however, following the administration of vitamin K_1, a marked increase in tyrosine phosphorylation of proteins of M_r 150–170, 120–130, 105–110, 67–70, and 55–60 kD was observed at both times. In the older, larger embryos, maximum effect on tyrosine phosphorylation was observed at vitamin K_1 doses 10-fold higher than that observed for the smaller day 12 embryos. The protein tyrosine phosphatase activity in brain of day 12 and day 18 embryos showed no significant change in the presence of vitamin K_1 (data not shown). In contrast, the protein–tyrosine kinase activity, as measured by the phosphorylation of synthetic random amino acid copolymer peptide substrates, increased up to threefold with increasing doses of vitamin K_1 from 0.45 μg to 45 μg (Fig. 2 *B*).

Tyrosine phosphorylation of c-Eyk is increased by vitamin K_1 supplementation. Western blot analysis using anti-c-Eyk antiserum confirmed the identity of the 105–110 kD band that exhibited modulation in its phosphotyrosine content in the presence of vitamin K_1 and warfarin (Fig. 1 *A*, 1 *C*, 2 *A*) as c-Eyk. Anti-Eyk immunoprecipitates of day 12 brain of the control and the vitamin K_1 pretreated embryos were then analyzed to establish whether the observed changes are resident in posttranslational tyrosine phosphorylation or due to increased synthesis of protein. Immunoprecipitates were first analyzed with anti-PY immunoblotting, and subsequently with anti-Eyk antiserum. While the c-Eyk immunoprecipitates from both the control and the vitamin K_1 pretreated embryos contained equivalent amounts of c-Eyk protein, a major increase in c-Eyk tyrosine phosphorylation was observed only in the vitamin K_1-treated embryos (Fig. 3).

Focal adhesion kinase ($pp125^{FAK}$), paxillin, and $pp60^{Src}$ are major components of vitamin K_1-dependent tyrosine phosphorylation cascade during chick embryogenesis. To identify other components of the vitamin K-induced tyrosine phosphorylation cascade, we focused on proteins that exhibited modulation in the presence of vitamin K_1 (Figs. 1 *A*, 2 *A*). Reprobe of days 12 and 18 brain blots (Figs. 1 *A* and 2 *A*) with anti-$pp125^{FAK}$, anti-paxillin, and anti-$pp60^{src}$ antibodies confirmed the identity of the 120–130 kD band as $pp125^{FAK}$, the 67–70 kD band as paxillin, and the 55–60 kD band as $pp60^{src}$. While anti-$pp125^{FAK}$ and anti-paxillin antibodies immunoprecipitated near equal amounts of $pp125^{FAK}$ and paxillin proteins from the brain and liver of both day 18 control and the vitamin K_1 pretreated embryos, only in the vitamin K_1 treated embryos were major increases in tyrosine phosphorylation of these proteins observed (Fig. 4 *A*, 4 *B*). Furthermore, anti-Src immune-complexes isolated from 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 (Fig. 5). The 150–170 kD protein that exhibited a dramatic change in tyrosine phosphorylation in the presence of vitamin K_1 or war-

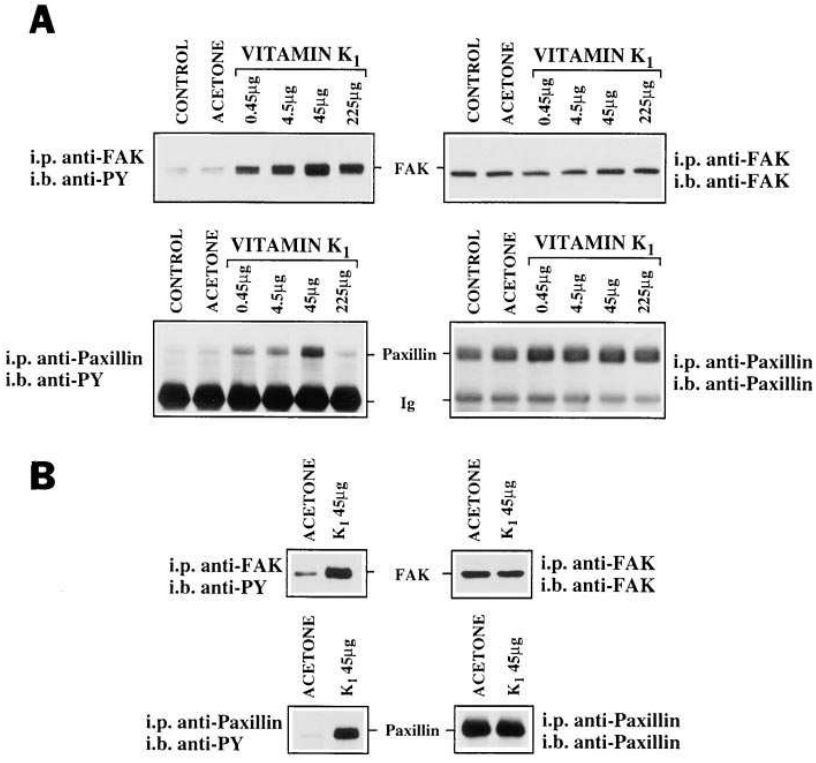

Figure 4. Identification of $pp125^{FAK}$ and paxillin as tyrosyl proteins modulated by vitamin K_1 in day 18 embryonic brain and liver. $pp125^{FAK}$ and paxillin were immunoprecipitated (i.p.) from detergent soluble lysates of brain (*A*) and liver (*B*) of day 18 embryos. Proteins were resolved by SDS-PAGE (7.5%), transferred to nitrocellulose, and analyzed by anti-PY immunoblotting (i.b.) to monitor vitamin K_1-induced changes in tyrosine phosphorylation. The blots were stripped and reprobed with anti-FAK or anti-paxillin antibodies for protein expression. Equivalent results were obtained in three individual experiments.

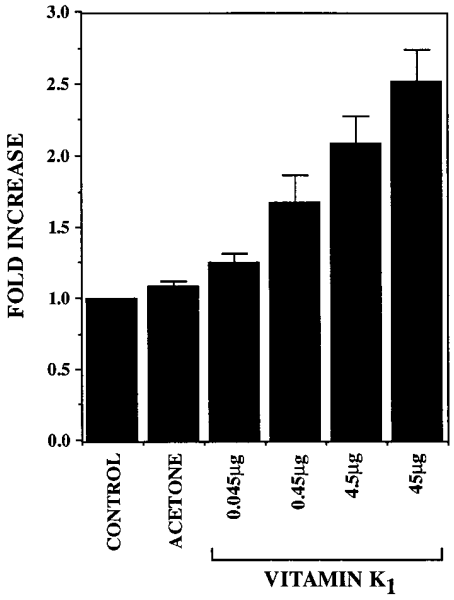

Figure 5. Anti-Src immune complexes isolated from the brain of day-18-old embryos treated with vitamin K_1 exhibited increased phosphorylation of $p34^{cdc2}$ peptides. $pp60^{src}$ protein was immunoprecipitated from the detergent soluble lysates of day 18 brain as described in Methods. Immune complexes were assayed for phosphorylation of a synthetic peptide (KVRKIGEGTYGVVKK) derived from amino acids 6–20 of $pp60^{src}$ with Tyr-19 replaced by Lys. The results are expressed as mean±SD of three independent experiments.

farin (Figs. 1, *A* and *C*, 2 *A*) is not PLCγ1—its identity remains to be determined.

Discussion

Protein tyrosine phosphorylation plays a pivotal role in the precise regulation of cell division, differentiation, and migration required for normal embryogenesis (29–33). Recent identification of vitamin K-dependent proteins as classical ligands for the Tyro family of RTKs (17–22) together with studies showing the role of vitamin K metabolism and function in two well characterized human birth defects, warfarin embryopathy (34) and vitamin K epoxide reductase deficiency (35), suggest the involvement of a vitamin K-dependent pathway(s) during embryonic development. In the present study we have demonstrated the existence of a vitamin K_1-sensitive tyrosine phosphorylation cascade in the developing chick embryo. That this cascade involves key intracellular proteins, including $pp125^{FAK}$, paxillin, and $pp60^{src}$, and responds to alteration in levels or metabolism of vitamin K_1, implies an important role for vitamin K_1-dependent signals in embryogenesis. Whether this vitamin K_1 effect is mediated by the γ-carboxylation of K-dependent proteins or by a mechanism independent of this cofactor activity has not been established.

The overall level of tyrosine phosphorylation is high in chick embryonic tissues during the early stages of development, decreases significantly during late embryogenesis, and is low or undetectable in the same tissues of the adult (10). In these studies, supplementation with vitamin K_1 both at an early stage (day 10) or at a later stage (day 16), significantly increased the tyrosine phosphorylation of several proteins in brain (Figs. 1 *A*, 2 *A*) and liver (data not shown). These tyrosine phosphorylated proteins, modulated in the presence of vitamin K_1, are similar to those previously shown to exhibit temporal changes in their level of tyrosine phosphorylation during chick embryonic development (10). Based on body weight, these effects were observed with doses of vitamin K_1 at or well below the usual prophylactic dose given to the full term human neonate. The effects of vitamin K_1 on protein–tyrosine phosphorylation were due neither to an effect on the overall expression of proteins (Fig. 1 *B*), nor to an effect on protein–tyrosine phosphatase activity (data not shown). Rather, vitamin K_1 supplementation caused an increase in protein–tyrosine kinase activity—up to threefold in day 16 brain (Fig. 2 *B*). Warfarin, an inhibitor of 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 embryos (Fig. 1 *C*); no inhibition was observed at higher vitamin K_1 levels sufficient to bypass the metabolic block. To our knowledge, this is the first demonstration of the inhibitory effects of warfarin on tyrosine phosphorylation during embryonic development and provides a possible explanation for the fetal toxicity of this drug (34).

c-Eyk, a 106K chicken counterpart of the Tyro 12 family of RTKs, exhibits a broad spatial and temporal expression during embryonic development (26). The 105–110 K band, which exhibited major alterations in phosphotyrosine content in the presence of vitamin K_1 and warfarin (Figs. 1, *A* and *C*, 2 *A*), was identified as c-Eyk by western blot analysis. Analysis of c-Eyk immunoprecipitates from the detergent soluble lysates of day 12 brain demonstrated that vitamin K_1 induced a major increase in its tyrosine phosphorylation in the absence of any change in the expression of c-Eyk protein (Fig 3). Although these studies suggest the involvement of c-Eyk in this vitamin K_1-induced tyrosine phosphorylation cascade, they do not exclude the possibility that other receptor systems may also mediate the effects of vitamin K_1 during embryonic development.

Protein expression and tyrosine phosphorylation of a cytoplasmic focal adhesion protein–tyrosine kinase ($pp125^{FAK}$) and its potential substrate paxillin are under developmental control (36, 37). Our studies demonstrate that vitamin K_1 supplementation induces tyrosine phosphorylation of $pp125^{FAK}$ and paxillin in brain and liver of the day 18 embryo without modifying the expression of these proteins (Fig 4 *A*, *B*). 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 (38). 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 (39). Consistent with these observations, the 55–60 K band that exhibited increased tyrosine phosphorylation in the presence of K_1 (Figs. 1 *A*, 2 *A*) was identified as $pp60^{Src}$ by Western blot analysis using anti-$pp60^{Src}$ antibody. Furthermore, anti-Src immune complexes isolated from embryos pretreated with vitamin K_1 showed up to a 2.5-fold increase in the phosphorylation of a synthetic peptide (Fig. 5). The concomitant increase in the tyrosine phosphorylation of $pp125^{FAK}$ as well as paxillin, an in vivo substrate of both $pp125^{FAK}$ and $pp60^{Src}$ (40), is consistent with the propagation of growth regulatory signals in a vitamin K_1-induced cascade (Figs. 4, *A* and *B*), and suggests that alterations in the levels of vitamin K_1 during embryogenesis may result in dysregulation of cell–cell or cell–matrix adhesion and other growth regulatory pathways.

Previous work from these laboratories relating to the possible regulatory effects of K_1 has recently been summarized (41). It is of more than passing interest that vitamin K_1 is maintained at low concentration in the human fetus and rises slowly to adult levels after birth in breast-fed babies unsupplemented with vitamin K. 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 (42). The inhibition of protein–tyrosine phosphorylation by warfarin is consistent with its known toxicity to the human fetus (34), as warfarin crosses the placenta and results in fetal death or skeletal anomalies similar to those described in congenital vitamin K epoxide reductase deficiency (35). The present studies suggest that vitamin K_1 is an important element in embryonic development and may explain, at least in part, the advantage of limiting its concentration in the mammalian and human fetus by a tightly regulated maternal/fetal placental gradient (6). This demonstration of a novel role of vitamin K_1 in the tyrosine phosphorylation cascade involving cytoskeletal proteins argues for the importance of tightly controlled levels of vitamin K_1 in the regulation of embryogenesis.

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