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Identification of chicken embryo kinase 5, a developmentally regulated receptor-type tyrosine kinase of the Eph family

Elena B. Pasquale
Department of Biology
University of California, San Diego
La Jolla, California 92093
La Jolla Cancer Research Foundation
La Jolla, California 92037

Chicken embryo kinase 5 (Cek5) is a transmembrane tyrosine kinase of the Eph family that was identified by screening a 10-d chicken embryo cDNA expression library with anti-phosphotyrosine antibodies. The extracellular region of Cek5 contains a cysteine rich N-terminal subdomain and a C-terminal subdomain mostly devoid of cysteines and comprising two repeats similar to fibronectin type III repeats. Immunoblotting experiments with anti-Cek5 polyclonal antibodies indicated that Cek5 is a membrane-associated 120-kDa protein containing intramolecular (but not intermolecular) disulfide bonds. Cek5 is already expressed in 2-d-old chicken embryos and is also expressed, at higher levels, later in development. In 10-d-old chicken embryos, Cek5 is expressed at substantial levels in nearly all the tissues examined, whereas in adult it is expressed predominantly in the brain. The expression of Cek5 in the brain gradually diminishes during embryonic development, whereas in the skeletal muscle of the thigh a sharp decrease in Cek5 expression was detected at the time of terminal muscle differentiation. Its wide tissue distribution throughout development and its sustained expression in adult brain suggest that Cek5 is an important component of signal transduction pathways, likely to interact with a widely distributed and important ligand, which is as yet unknown.

Introduction

It is becoming increasingly clear that protein tyrosine kinases play crucial roles in the cascade of events involved in signal transduction during embryonic development (Pawson and Bernstein, 1990). Evidence for the fundamental importance of protein tyrosine kinases during development includes the following: 1) protein tyrosine phosphorylation, resulting from the enzymatic activity of protein tyrosine kinases, is detected in embryonic tissues and is particularly extensive during certain stages of development (Maher and Pasquale, 1988); 2) in agreement with this, a large number of different tyrosine kinase genes are found to be expressed during embryonic development (Garofalo and Rosen, 1989; Girbau *et al.*, 1989; Pasquale and Singer, 1989; Musci *et al.*, 1990; Pasquale, 1990; Pawson and Bernstein, 1990 and references therein); and 3) mutations in these kinase genes often cause major developmental defects (Pawson and Bernstein, 1990 and references therein).

Protein tyrosine kinases with the structure of transmembrane receptors are likely to represent important components of developmental signaling pathways. Their structure suggests that receptor tyrosine kinases represent transducers of signals across the plasma membrane, from the cell exterior to the cytoplasm. They generally are type I integral membrane proteins (Singer, 1990) containing a single transmembrane segment that connects a ligand-binding extracellular domain with a cytoplasmic domain that has catalytic activity. The interaction of the external domain with the ligand, often a growth factor, stimulates the enzymatic activity of the intracellular catalytic domain and, as a consequence, the phosphorylation on tyrosine of cytoplasmic substrates occurs (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990).

To understand how receptor tyrosine kinases function in signal transduction during development, it is necessary to identify and characterize their genes. Such a project was first undertaken by Pasquale and Singer (1989) at a time when few of the protein tyrosine kinases expressed during embryonic development were known. An approach was devised that would not be biased toward the identification of tyrosine kinases similar in their primary sequence to known ones and that would favor the identification of the most abundant and widely expressed embryonic tyrosine kinases. Presumably these would be tyrosine kinases playing fundamental roles in developmental processes. As described (Pas-

quale and Singer, 1989), the approach used consisted of choosing a cDNA expression library that was thought likely to contain a large pool of developmentally important tyrosine kinase genes. Because protein tyrosine phosphorylation was known to be extensive in many tissues from 8- to 10-d-old chicken embryos (Maher and Pasquale, 1988), a cDNA library prepared from whole 10-d embryos was used. The cDNA clones encoding enzymatically active tyrosine kinase fragments were identified by probing the library with anti-phosphotyrosine antibodies. Among the protein tyrosine kinases identified in an initial screening, five were previously unknown and had the characteristics of integral membrane receptors. They were designated chicken embryo kinase (Cek) 1–5.

Cek1, Cek2, and Cek3 have been described (Pasquale and Singer, 1989; Pasquale, 1990) and belong to the previously uncharacterized family of the fibroblast growth factor (FGF) receptor (Lee *et al.,* 1989; Ullrich and Schlessinger, 1990). The Cek1, Cek2, and Cek3 receptor tyrosine kinases presumably mediate some of the diverse biological effects of the FGF family of growth factors (Burgess and Maciag, 1989) and are therefore likely to represent essential molecules in extremely important developmental signaling pathways. Their identification demonstrated the value of the approach chosen.

Cek4 and Cek5 are related to each other and to the Eph tyrosine kinase (Hirai *et al.,* 1987). The Cek5 cDNA and deduced amino acid sequences are reported here; Cek4 will be described elsewhere. The kinases belonging to the Eph family have not yet been extensively characterized. Thus the molecules (ligands) that are presumed to interact with their extracellular domains, as well as their intracellular substrates, are unknown. The characterization of the Cek5 kinase expression during development, obtained using specific polyclonal antibodies and described here, provides evidence that Cek5 represents a component of signal transduction pathways that are activated throughout embryonic development and are also functional in adult brain.

Results

DNA sequence and deduced amino acid sequence of Cek5

Among the cDNA clones isolated by screening a 10-d chicken embryo expression library with anti-phosphotyrosine antibodies (Pasquale and Singer, 1989), three encoded portions of the same protein, which was designated Cek5. The largest clone (clone 10Q) was completely sequenced (nucleotides 494–3228 in Figure 1). Additional Cek5 clones, extending further in the 5′ region, were searched for by using clone 10Q as a probe to rescreen the 10-d chicken embryo library. One of the new cDNAs isolated was 4.0 kb long and contained *Sal* I, *Sma* I, *Hin*dIII, and *Sst* I sites corresponding to those in the 10Q clone. The 5′ end of the 4.0-kb clone was sequenced (nucleotides 1–525 in Figure 1). Nucleotides 1–493 (Figure 1) were not present in clone 10Q, and nucleotides 494–525 (Figure 1) were identical to nucleotides 2–33 of clone 10Q. The 4.0-kb clone also contained ~820 additional nucleotides at the 3′ end (not shown). This region included a polyadenylation signal that, however, was not followed by a poly(A) tail.

The Cek5 DNA sequence and deduced amino acid sequence are shown in Figure 1. Two ATG codons, in the same reading frame, are located within the first 40 nucleotides (Figure 1). Although the second ATG codon is in a more favorable context for translation initiation (Kozak, 1989), the first ATG is the one corresponding to the initiation site of homologous tyrosine kinases (Figure 2b) (Pasquale, unpublished results). It cannot be ruled out that both ATGs may be used for translation initiation (Kozak, 1989) and that two different proteins are produced, one of which lacks the first seven amino acids of the signal peptide.

An open reading frame extends for 926 (or 919) amino acids. The presence of two hydrophobic stretches of amino acids, presumably representing a cleavable signal peptide (residues 1–26 or 8–26) (von Heijne, 1986) and a transmembrane region (residues 557–573), suggests that Cek5 is a type Ia integral membrane protein (Singer, 1990). The predicted molecular weight of Cek5, after removal of the signal peptide, is 108 000. The region N-terminal to the putative transmembrane domain is presumably extracellular and can be divided into two subdomains. The N-terminal subdomain, comprising residues 27–328, contains numerous cysteines. The C-terminal subdomain of the extracellular region, comprising residues 329–556, is mostly devoid of cysteines and contains two repeats homologous to fibronectin type III repeats (Figure 2a) (Skorstengaard *et al.,* 1986; Ruoslahti, 1988). These repeats of ~100 amino acids are characterized by the presence of conserved aromatic and hydrophobic residues (Figure 2a) and lack conserved cysteine residues (Norton *et al.,* 1990; Patthy, 1990). Repeat 2 (residues 440–539) is located in close proximity to the transmembrane region. The transmembrane region is immediately followed by three

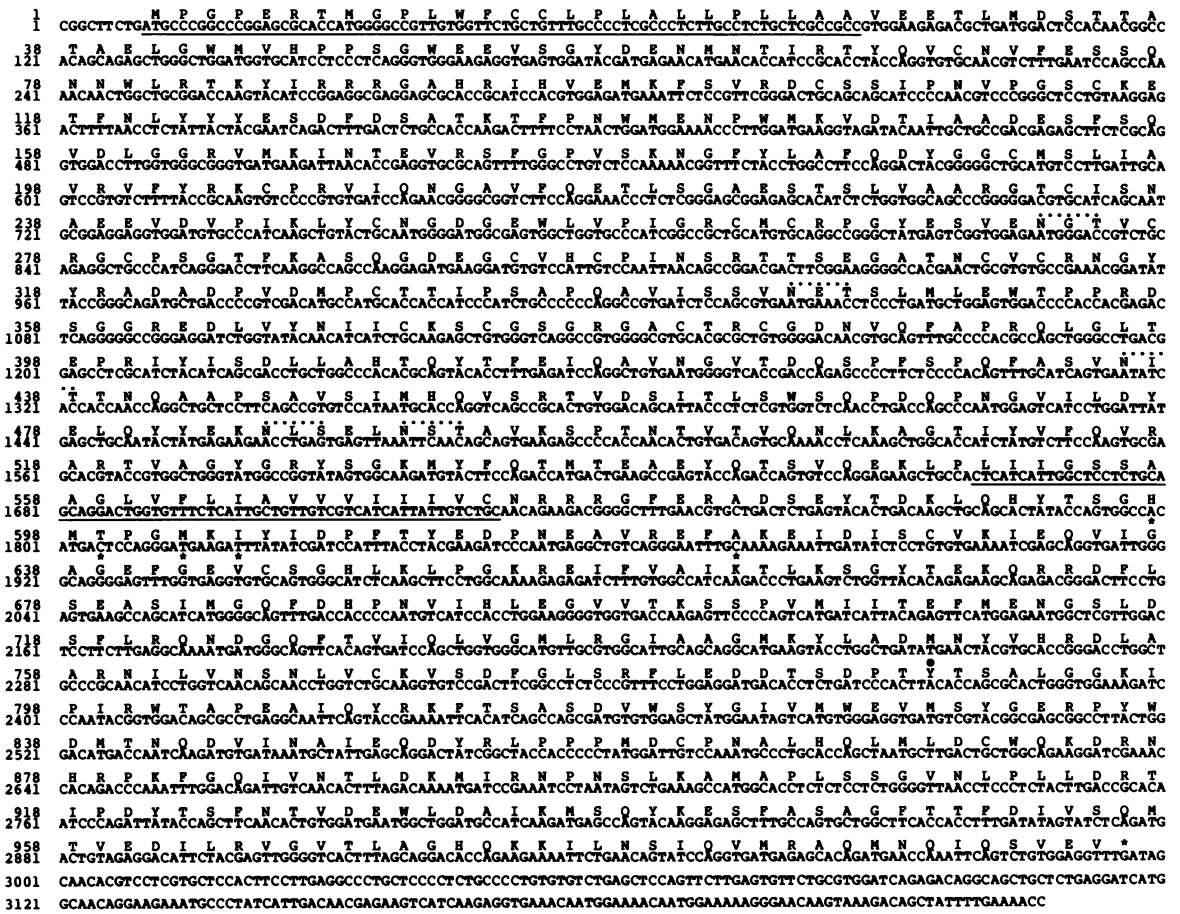

Figure 1. **Nucleotide sequence and predicted amino acid sequence of the longest Cek5 cDNA clone isolated.** The presumed signal-peptide sequence and the single putative transmembrane domain are underlined, the possible sites of N-glycosylation are marked by dotted overlines, some of the residues involved in ATP binding are labeled by stars above the amino acid symbols, and the tyrosine putative site of autophosphorylation is indicated by a filled circle. Nucleotides and amino acids are numbered at left. The nucleotides are numbered starting from the first residue after the *Eco*RI site of lambda gt11.

basic residues, likely to represent a stop-transfer sequence.

The cytoplasmic region of Cek5, C-terminal to the transmembrane domain, contains all the residues that are diagnostic of protein tyrosine kinase catalytic domains (Hanks *et al.,* 1988). Some of these residues are marked in Figures 1 and 2b. A juxtamembrane region of 54 amino acids separates the transmembrane region from the catalytic domain, and a C-terminal tail of 99 amino acids follows the kinase domain.

Comparison with the sequences of other receptor-type protein tyrosine kinases indicates that Cek5 is related to the Eph kinase (Hirai *et al.,* 1987). In Figure 2b the sequence of Cek5 is compared with those of Eph and of the two other known protein tyrosine kinases related to Eph: Elk (Letwin *et al.,* 1988) and Eck (Lindberg and Hunter, 1990). The overall amino acid sequence identity of Cek5 with Eph is 40% and with Eck is 48%. Only the partial amino acid sequence shown in Figure 2b is available for Elk. This portion of Elk is 83% identical to the corresponding region of Cek5. Figure 2b shows that, in the extracellular region, all the cysteines (″) (with the exception of Eph Cys_{171}) are conserved in Cek5, Eck, and Eph. Also conserved are the aromatic and hydrophobic residues characterizing the region of homology to fibronectin (see also Figure 2a). The catalytic domains (corresponding to Cek5 residues 628–896) are the regions of closest similarity among the members of the Eph family.

Immunological detection of the Cek5 protein

To characterize the developmental expression of Cek5, we prepared specific polyclonal antibodies in two different ways. In one case, the portion of Cek5 encoded by clone 10Q (amino

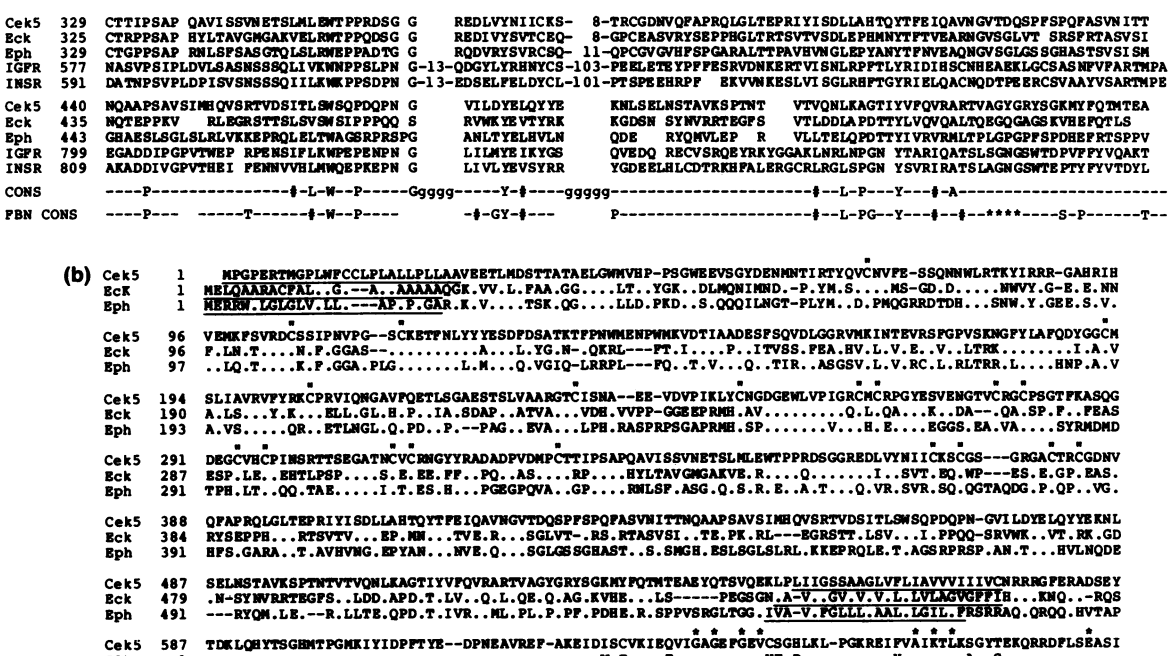

Figure 2. **Relationships of Cek5 with other proteins.** (a) Alignment (by eye) of the two repeats homologous to fibronectin type III repeats (Skorstengaard *et al.,* 1986) found in Cek5, Eck (Lindberg and Hunter, 1990), Eph (Hirai *et al.,* 1987), and insulin and insulin-like growth factor 1 receptors (Ullrich *et al.,* 1986). Gaps were introduced to maximize the alignment. The numbering is as in Figure 1 and in Lindberg and Hunter (1990), Hirai *et al.* (1987), and Ullrich *et al.* (1986). The presence of one of the hydrophobic residues L, I, or V in ≥90% of the repeats is marked by #. Conserved hydrophobic residues and residues conserved in ≥70% of the 15 fibronectin type III repeats (Skorstengaard *et al.,* 1986) are also indicated for comparison (FBN CONS). The sequence RGDS, only present in repeat 10 of fibronectin, is marked by ****. (b) Comparison of the amino acid sequences of Elk (Letwin *et al.,* 1988), Eck (Lindberg and Hunter, 1990), and Eph (Hirai *et al.,* 1987) with that of Cek5. The sequences were aligned using the program DFALIGN (Feng and Doolittle, 1987). Dots replace residues in Elk, Eck, and Eph that are identical to the corresponding residue in Cek5. Hyphens represent gaps introduced in the sequences to aid the alignment. Signal peptide sequences and putative transmembrane domains are underlined. The conserved cysteines in the extracellular domains are indicated ('). Asterisks mark residues that are conserved in protein-tyrosine kinase catalytic domains (Hanks *et al.,* 1988). ●, indicates the tyrosine that is the putative acceptor for autophosphorylation (Hanks *et al.,* 1988).

acids 167–926 in Figure 1), expressed in bacteria as a β-galactosidase fusion protein, was used as an antigen and for affinity purification of the immune serum. Alternatively, rabbits were immunized with a peptide corresponding to the 10 carboxy-terminal amino acids of Cek5. In immunoblotting experiments, both types of anti-Cek5 antibodies labeled a single protein band of ~120 kDa in extracts from 10-d chicken embryonic brain (Figure 3, a and b, BR).

The anti-Cek5 fusion protein antibodies recognize specifically the Cek5 kinase but not other proteins containing a homologous tyrosine kinase catalytic domain. In fact, anti-Cek5 antibodies eluted from the 120-kDa region of an immunoblot of 10-d embryonic brain recognize the Cek5-β-galactosidase fusion protein but not a Cek1-β-galactosidase fusion protein (Pasquale and Singer, 1989), which contains a catalytic domain 35% identical, at the amino acid level,

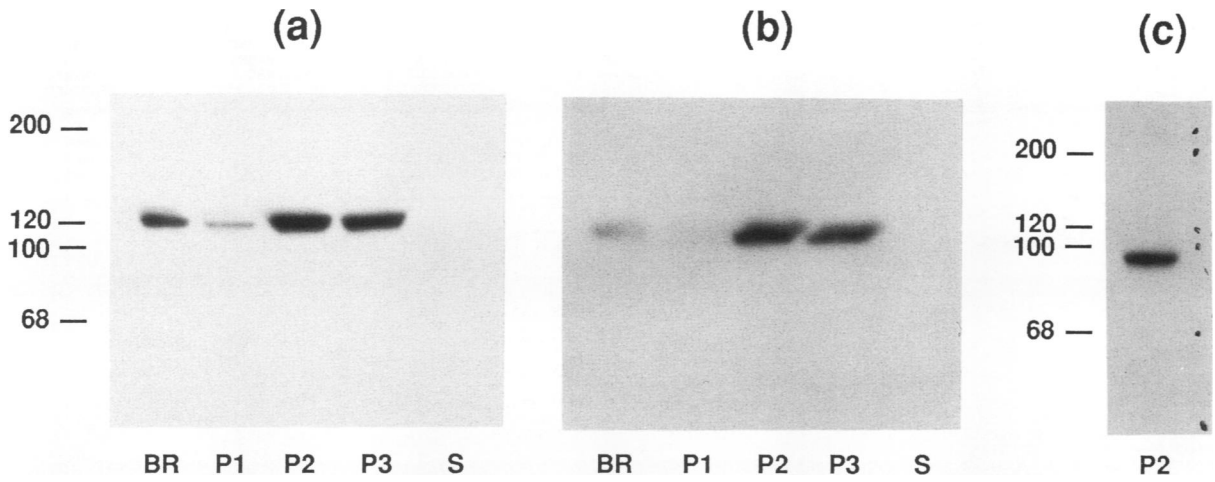

Figure 3. **Immunoblot of various subcellular fractions from 10-d chicken embryonic brain with anti-Cek5 polyclonal antibodies: (a) and (c) anti-Cek5-β-galactosidase fusion protein antibodies and (b) anti-Cek5 C-terminal peptide antibodies.** The gel in c was run under nonreducing conditions (without β-mercaptoethanol in the sample buffer). BR, 10-d brain; P1, 1000 × *g* pellet; P2, 17 000 × *g* pellet; P3, 200 000 × *g* pellet; S3, 200 000 × *g* supernatant. Equal amounts of protein were loaded in all of the lanes. Molecular weight markers ($\times 10^{-3}$) are indicated at left in this and Figures 4–8.

to that of Cek5 (not shown). The anti-peptide antibodies also appeared to be specific for Cek5. They labeled the bacterially expressed Cek5 fusion protein and were detectable in the rabbit serum only after repeated immunizations with the peptide (not shown).

The apparent molecular weight of Cek5 is somewhat higher than the molecular weight calculated on the basis of the amino acid sequence. This is presumably due to glycosylation of the extracellular domain, because five possible sites for N-linked glycosylation are present in the sequence of the extracellular domain of Cek5 (Figure 1). In an immunoblot of 10-d embryonic brain, obtained under nonreducing conditions with the anti-fusion protein antibodies, the apparent molecular weight of the Cek5 protein is ~90 000 (Figure 3c). The lower apparent molecular weight obtained in the absence of reducing agents suggests the presence of intramolecular disulfide bonds and is consistent with the presence of numerous cysteines in the extracellular portion of the Cek5 protein. This experiment also showed that in vivo the Cek5 molecules are not covalently linked to one another or to other molecules through disulfide bonds.

To examine the subcellular distribution of Cek5, we homogenized and fractionated a 10-d chicken embryonic brain by differential centrifugation (Figure 3, a and b). The following fractions were obtained: a 1000 × *g* pellet (P1), containing nuclei and unbroken cells; a 17 000 × *g* pellet (P2), and a 200 000 × *g* pellet (P3), both containing membranes; and a supernatant (S3), containing soluble proteins. Both types of anti-Cek5 antibodies described above detected Cek5 almost exclusively in the two membrane fractions, equally distributed between P2 and P3 (compare Figure 3, a and b). The membrane association of Cek5 is in agreement with its amino acid sequence, diagnostic of an integral membrane protein.

Because the anti-fusion protein antibodies produced stronger labeling in immunoblotting experiments (Figure 3), they were used for all subsequent experiments (Figures 4–8). However, the antipeptide antibodies were used to reproduce some experiments (not shown) and produced similar results.

Characterization of the developmental expression of the Cek5 kinase

In Figure 4a the expression of Cek5 in different tissues from a 10-d chicken embryo was examined. The results indicate that Cek5 is nearly ubiquitous at this stage of development. It is most abundant in 10-d embryonic brain and is also expressed at high levels in kidney, lung, intestine, gizzard, and thigh. It is detectable at lower levels in liver, heart, and lens, but not in whole blood. In adult chicken tissues (Figure 4b), Cek5 is most abundant in brain; however, it is present at lower levels than in embryonic brain (Figures 4b and 5). Cek5 is clearly detectable in adult liver and, at lower levels, in intestine and gizzard. Very low levels of Cek5 are detectable in adult lung, thigh, and heart, but only after greatly increasing the exposure of the autora-

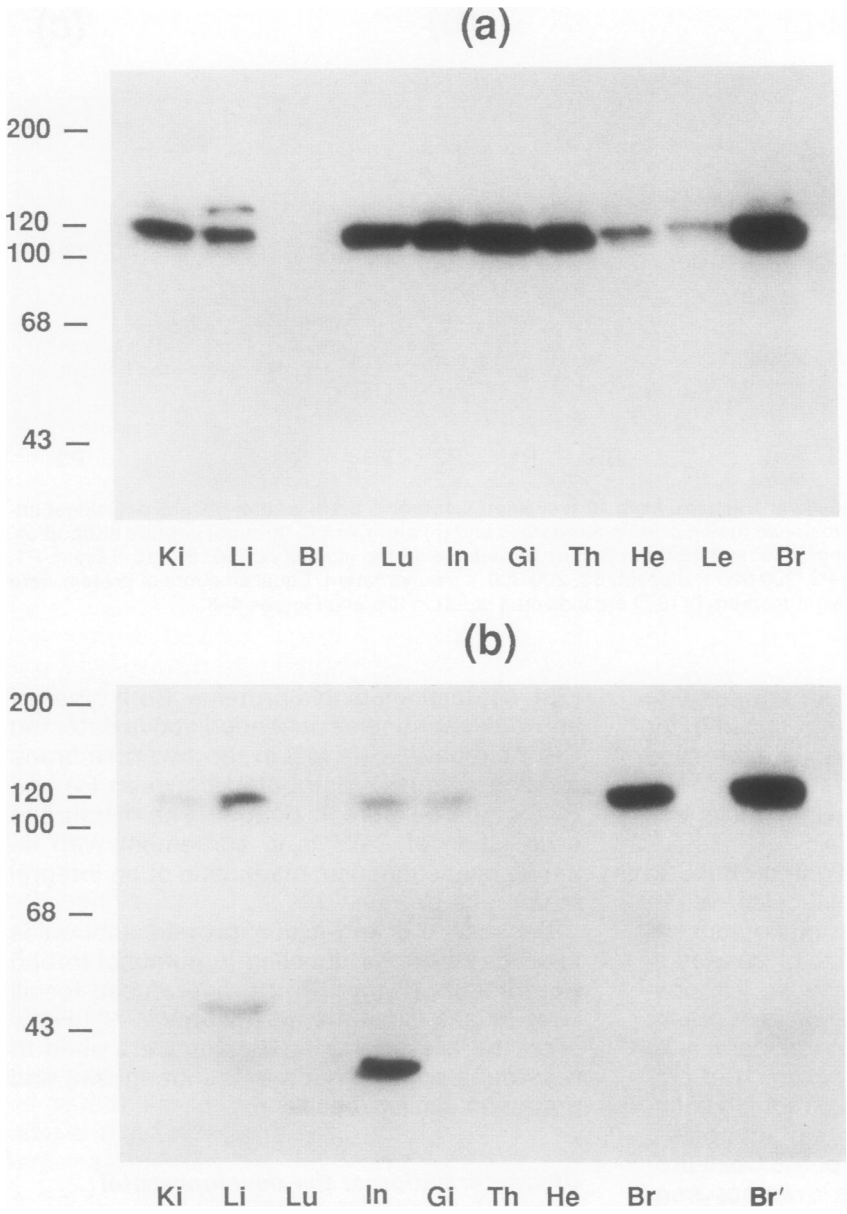

Figure 4. **Immunoblot with anti-Cek5 anti-fusion protein antibodies of various chicken 10-d embryonic (a) and adult (b) tissues.** Ki, kidney; Li, liver; Bl, blood; Lu, lung; In, intestine; Gi, gizzard; Th, thigh; He, heart; Le, lens; and Br, brain. Br′ in (b) indicates 10-d brain (shown for comparison).

diographs (not shown and Figure 6a inset). The nature of the protein band of ~140 kDa in embryonic liver and of the 35-kDa-band in adult intestine is not known.

Comparison of Figure 4, a and b, indicates that the expression of Cek5 is substantially lower in adult tissues than in the corresponding tissues from 10-d chicken embryos. The expression of Cek5 during development was examined in greater detail in two tissues: brain and skeletal muscle of the thigh. In brain tissue (Figure 5), the expression of Cek5 is high and relatively constant in embryos from 7 to ~12 d old. The abundance of Cek5 then gradually decreases up to the time of hatching. In adult brain, Cek5 is still easily detectable but less abundant than during development. In the skeletal muscle of the thigh, the regulation of the expression of Cek5 during development is different from that in brain (Figure 6a). Cek5 is present at about the same level in skeletal muscle of 7- to 15-d-old embryos. Between days 15 and 17 of development in ovo, however, the level of Cek5 expression decreases sharply and remains low until hatching. Remarkably, the total protein composition of the thigh muscle also changes markedly between days 15 and 17 of development in ovo (Figure 6b), presumably due to the increased expression of some muscle-specific proteins, such as myosin (arrow in Fig-

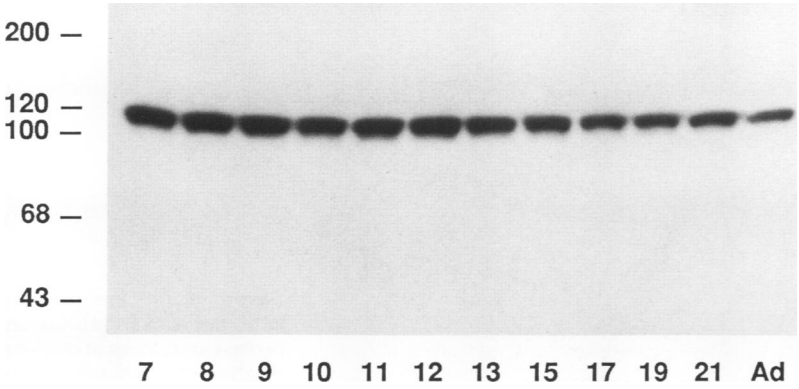

Figure 5. **Immunoblot with anti-Cek5 anti-fusion protein antibodies of chicken brain during embryonic development (days of development in ovo are indicated at the bottom) and in adult (Ad).**

ure 6b) (Hermann, 1952; Fishman, 1967). The expression of Cek5 in adult muscle is extremely low and is only detectable after longer exposure of the autoradiograph (Figure 6a, insert). Immunoblots of skeletal muscle during development also consistently reveal the faint labeling of a 65-kDa protein band from 12 to 21 d of development in ovo (Figure 6a, arrow on the right) and of a 40-kDa protein band from 7 to 15 d (Figure 6a, arrow on the left). The possible significance of this labeling is discussed below (Discussion).

To examine the expression of the Cek5 tyrosine kinase earlier in development, we prepared extracts from 2- to 10-d-old whole embryos and examined them by immunoblotting (Figure 7). Cek5 is already detectable in 2-d-old embryos and is considerably more abundant in 3-d-old embryos. The expression of Cek5 is higher and does not change significantly between days 4 and 10 of development in ovo.

Tyrosine kinase activity of Cek5

Cek5 was identified by detecting its tyrosine kinase activity in bacterial plaques with anti-phosphotyrosine antibodies. As described above, sequence homology also suggests that Cek5 contains a functional tyrosine kinase catalytic domain. Furthermore, as shown in Figure 8, Cek5 isolated from 11-d chicken embryonic brain displays tyrosine kinase activity in vitro and autophosphorylates on tyrosine residues, after incubation in a buffer containing ATP (Figure 8, lane 2). Cek5 isolated from embryonic tissues was found not to contain any detectable phosphotyrosine before undergoing the in vitro autophosphorylation reaction (not shown). Accordingly, the pattern of tyrosine phosphorylation in brain extracts is not affected by the removal of Cek5 (Figure 8, compare lanes 3 and 4).

Discussion

Structural features of Cek5

Cek5 is one of the receptor tyrosine kinases discovered by a procedure designed to identify protein tyrosine kinases involved in developmental signal transduction pathways. Its primary structure indicates that Cek5 is a member of a family of receptor tyrosine kinases for which the Eph kinase is the prototype (Hirai *et al.,* 1987). The only other Eph-related kinase for which the entire amino acid sequence has been previously determined is Eck (Lindberg and Hunter, 1990). Therefore, the determination of the full-length sequence of Cek5 allows the identification of some of the specific features characterizing the overall structure of the Eph family of protein tyrosine kinases. These include a unique distribution of 20 conserved cysteines in the extracellular regions. Seventeen of these cysteines are clustered in a cysteine-rich region, separated from the transmembrane domain by a cysteine-free stretch of ~170 amino acids. Furthermore, the regions separating the transmembrane and putative ATP binding regions of the kinases in the Eph family have a characteristic length of 61–70 amino acids, shorter than in the FGF receptor family (83–89 amino acids) and longer than in most other receptor tyrosine kinases (~50 amino acids). The catalytic domains of the Eph-related kinases are not interrupted by kinase insert regions (Hanks *et al.,* 1988) and are followed by C-terminal tails of 89–99 amino acids.

The amino acid sequence of Cek5 suggests the presence of a functional tyrosine kinase catalytic domain. Cek5 also contains potential sites of autophosphorylation, such as a conserved tyrosine within the catalytic domain (Tyr_{789}) (Hanks *et al.,* 1988) and a tyrosine in the C-terminal tail that is conserved within the Eph family (Tyr_{939}). Accordingly Cek5, expressed in

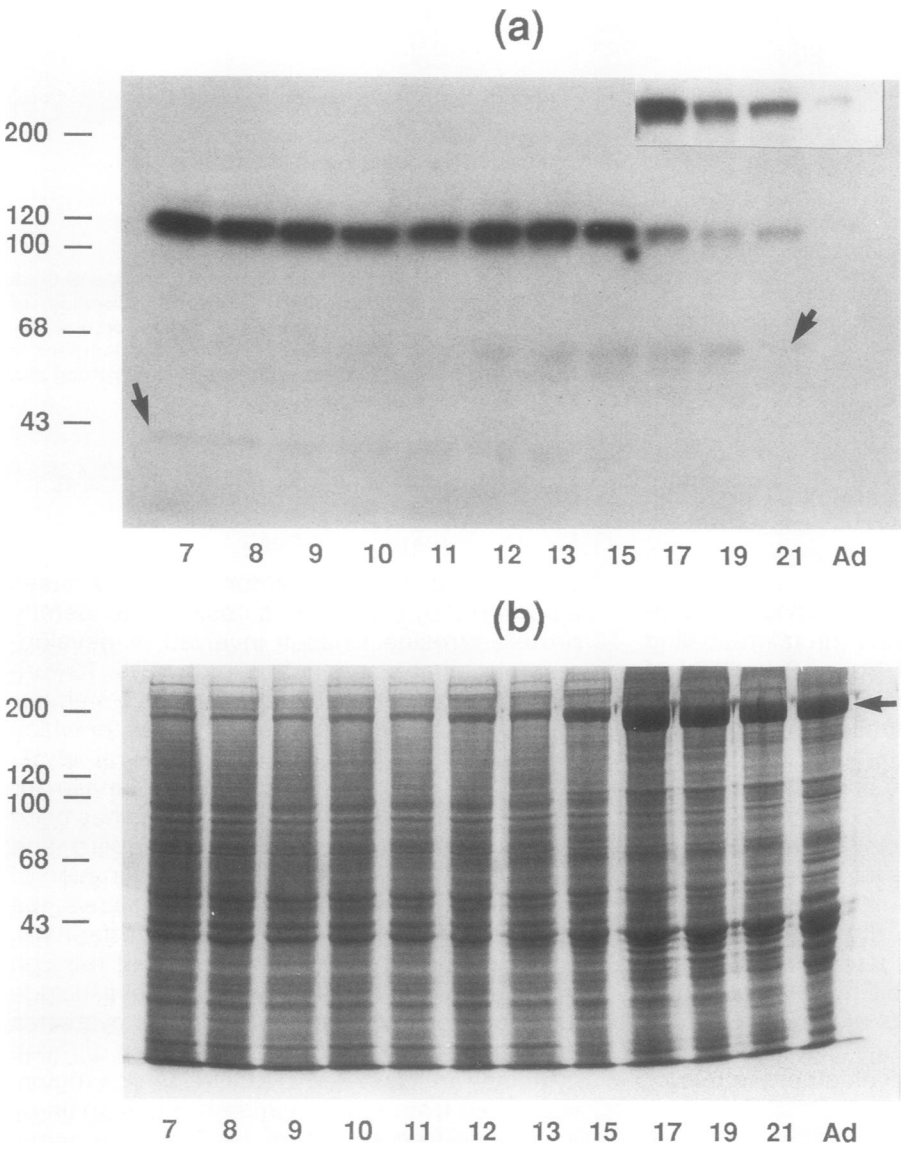

Figure 6. **(a) Immunoblot with anti-Cek5 anti-fusion protein antibodies of chicken thigh during embryonic development and in adult. (b) Coomassie blue-stained gel showing the pattern of proteins expressed in embryonic and adult thigh.** Days of embryonic development are indicated at the bottom; Ad indicates adult thigh. The inset in a shows a longer exposure of the 120-kDa Cek5 band in lanes 17, 19, 21, and Ad. Cek5, which is not detectable in adult in a, is detectable as a faint band in the inset. The arrow on the right indicates a 65-kDa band labeled in 12- to 21-d-old thigh. The arrow on the left indicates the 40 kDa and labeled in 7- to 15-d-old thigh. The arrow in b indicates one of the protein bands, presumably containing myosin (Hermann, 1952; Fishman, 1967), the expression of which is sharply increased in thigh skeletal muscle between 15 and 17 d of development in ovo. The gel lanes in b contain ~25% as much protein as the gel lanes in a.

bacteria as a Cek5-β-galactosidase fusion protein, is capable of catalytic activity and autophosphorylation. This allowed its identification with anti-phosphotyrosine antibodies. Furthermore, Cek5 isolated from 11-d embryonic brain displays tyrosine kinase activity and autophosphorylates in vitro (Figure 8). These observations suggest that Cek5 also has intrinsic tyrosine kinase activity in vivo.

Relationships of Cek5 with other proteins

Portions of the extracellular region of Cek5 show interesting homologies with other proteins. Although these homologies involve amino acid residues that are conserved in Eck and Eph, they have not been previously reported. It is important to identify these conserved sequence motifs because they are presumably involved in the interactions, as yet uncharacterized, of the Eph-related tyrosine kinases with ligand molecules accessible to the external surface of the plasma membrane. Extracellular domains similar to that of Cek5, consisting of an N-terminal cysteine-rich segment and a C-terminal segment containing repeats homologous to the type III repeats of fibronectin, have been described for many proteins mediating protein–protein interactions. These include various members of the growth hormone/prolactin receptor family and a number of developmentally regulated neural cell adhesion molecules (Norton *et al.*, 1990; Patthy, 1990 and references therein). The insulin and insulin-like growth fac-

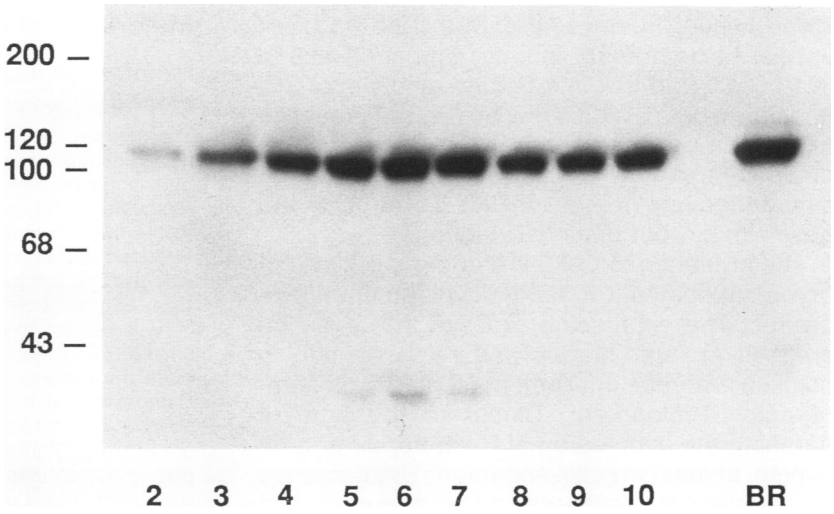

Figure 7. **Immunoblot with anti-Cek5 anti-fusion protein antibodies of whole chicken embryonic extracts.** The age of embryonic development, in days, is indicated at the bottom. Ten-day embryonic brain (BR) is also shown for comparison.

tor 1 receptors also contain two fibronectin type III repeats (Figure 2b), which have not been previously reported. In the latter receptors, repeat 1 is interrupted by a long inserted sequence containing the cleavage site between the α- and β-receptor subunits (Figure 2b), and repeat 2 is located just N-terminal to the transmembrane domain. Seven fibronectin type III repeats are also present in another receptor tyrosine kinase, sevenless (Norton *et al.,* 1990). Six C-X-C or C-X-X-C sequences are present in the Cek5 extracellular region. It is not known whether these sequences are involved in complex formation with metal ions, in analogy with their presumed function in several metal-binding proteins (Kojima, 1976 and references therein; Furey *et al.,* 1986).

Inspection of Figure 2 reveals an 83% identity at the amino acid level between Cek5 (a chicken protein) and Elk (Letwin *et al.,* 1988) (a rat protein) throughout the catalytic and C-terminal regions (the only regions for which the Elk sequence is available). However, the recent identification of a 162-bp portion of the rat homologue of the Cek5 cDNA, which is distinct from the corresponding Elk cDNA, indicates that Cek5 and Elk represent two different gene products (Lai and Lemke, 1991).

Nucleotides 2209–2388 of Cek5 (Figure 1) are identical, with the exception of two nucleotides, to a 180-bp portion of the 373-bp chicken genomic sequence TKR11, which was identified with a v-*fps* probe and thought to be conserved among avian and mammalian species (Foster *et al.,* 1986). The TKR11 cDNA presumably represents a portion of the Cek5 gene or of a Cek5 allele, and the lack of homology outside the 180-bp region may be explained with the presence of introns in the TKR11 DNA.

Developmental expression of the Cek5 protein

Its wide tissue distribution and abundant expression suggest that Cek5 has important physiological functions. The expression during development of Eph, Elk, and Eck has not been investigated. Thus, it is not known whether these members of the Eph family are equally abundant in embryonic tissues or whether Cek5 is the most ubiquitous of the Eph-related kinases and the others have a more limited distribution and, presumably, more restricted functions. mRNAs corresponding to Eph, Elk, and Eck have been detected in rat adult tissues (Letwin *et al.,* 1988; Maru *et al.,* 1988; Lindberg and Hunter, 1990). If the presence of mRNA reflects the presence of the corresponding protein, these data suggest that several of the kinases in the Eph family can be expressed in the

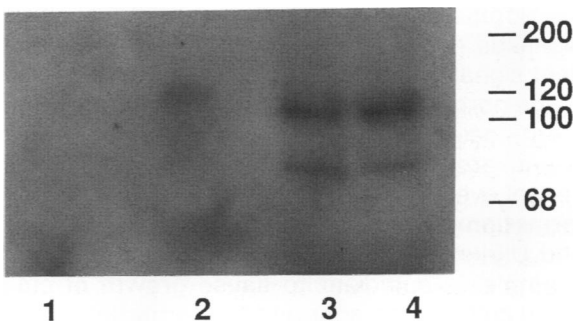

Figure 8. **Immunoblot with antiphosphotyrosine antibodies of: lane 1, control immunoprecipitation; lane 2, immunoprecipitated Cek5; lane 3, 11-d embryonic brain extract after immunoprecipitation of Cek5; lane 4, 11-d embryonic brain extract after control immunoprecipitation.** The immunoprecipitates in lane 1 and 2 were incubated under conditions that allow in vitro phosphorylation (see Materials and Methods).

same tissue. For example, both Eph and Eck appear to be present in lung; Eph and Cek5 in liver; Cek5 and Eck in intestine; and Cek5, Elk, and Eck in brain. Within one tissue, the presence of the Eph-related kinases in specific cell types could be mutually exclusive, or they could be simultaneously present in the same cells and have similar, but distinct, functions.

The presence of Cek5 in 2-d-old chicken embryos implicates it in early developmental processes. The expression of Cek5, however, increases at later stages and remains high in chicken embryos until at least the 10th d of embryonic development. Closer to the time of hatching the expression of Cek5 appears to diminish, at least in brain and thigh. The decrease in Cek5 receptor expression that occurs during skeletal muscle differentiation correlates with changes in muscle protein composition (Hermann, 1952; Fishman, 1967). This is similar to the loss of FGF and epidermal growth factor receptors that has been shown to be a specific phenotype acquired during skeletal muscle differentiation (Olwin and Hauschka, 1988, 1990). In immunoblots of skeletal muscle during development, the anti-Cek5 antibodies faintly label, in addition to the 120-kDa Cek5 protein, two protein bands of 65 and 40 kDa. This labeling, if specific, suggests the existence in skeletal muscle of proteins immunologically related to Cek5 with lower apparent molecular weights. Their expression, very limited and restricted to some developmental stages (Figure 6a), could be explained by the presence of alternatively spliced forms of the Cek5 mRNA.

Because Cek5 is such a prevalent molecule in the embryo and in some adult tissues, overexpression, decreased expression, or mutations of the Cek5 gene are likely to produce major developmental defects in the embryo as well as diseases due to malfunctions in Cek5-dependent signaling pathways in the adult. Alterations of the normal responses to the cellular environment, caused by defects in tyrosine kinase genes, are thought in many cases to represent causal events in cell transformation and tumor formation (Hunter and Cooper, 1986; Yarden and Ullrich, 1988). Overexpression of Eph, for example, is sufficient to cause growth of cultured cells in soft agar and the formation of tumors in nude mice (Maru *et al.*, 1990). Thus, the identification and characterization of protein tyrosine kinases important in developmental processes, such as presumably Cek5, represents a contribution to the understanding not only of developmental processes, but also of oncogenic transformation.

Materials and methods

Isolation and characterization of cDNA clones

The method for screening a cDNA expression library with anti-phosphotyrosine antibodies and the procedures for sequencing have been described (Pasquale and Singer, 1989; Pasquale, 1990; Lindberg and Pasquale, 1991). In addition to the use of suitable restriction enzyme sites, a double-stranded exonuclease kit (Pharmacia LKB, Piscataway, NJ) was used to produce a set of pBluescript (Stratagene, La Jolla, CA) deletion clones carrying Cek5 cDNA inserts of various lengths to be used for sequence analysis. Several oligodeoxynucleotides were also used as primers to complete the sequencing of both strands of the Cek5 cDNA. Clones extending into the 5′ region of the Cek5 cDNA were searched for by screening a 10-d chicken embryo lambda gt11 library (Clontech, Palo Alto, CA) with clone 10Q (comprising nucleotides 494–3228 in Figure 1). Clone 10Q was the longest clone previously isolated from the same library by probing with antiphosphotyrosine antibodies. The screening with the 10Q DNA probe was performed using a nonradioactive DNA labeling and detection method (Boehringer Mannheim, Indianapolis, IN) and high-stringency conditions for hybridization (50% formamide, 0.75 M NaCl, 0.075 M sodium citrate [pH 7.0], 0.06% Ficoll, 0.06% polyvinylpyrrolidone, 0.06% bovine serum albumin [BSA], 0.5% sodium dodecyl sulfate [SDS], with 200 μg of sheared salmon sperm DNA per ml at 37°C). Three hundred thousand plaques were screened to isolate the 4.0-kb clone that contributed the first 493 nucleotides to the final Cek5 sequence.

Preparation of antibodies specific for Cek5

Clone 10Q was subcloned in the *Eco*RI site of the expression vector pEX2 (Boehringer Mannheim) to obtain a high level of expression of the β-galactosidase-Cek5 fusion protein. As described (Pasquale and Singer, 1989), the insoluble material obtained after sonication of bacteria expressing pEX2 plasmids, which contained all of the β-galactosidase-Cek5 fusion protein as the most prominent component, was mixed with adjuvant and injected into rabbits. Antibodies specific for the Cek5 kinase were purified on an affinity column prepared by coupling the β-galactosidase-Cek5 fusion protein, eluted from preparative SDS/polyacrylamide gels, to Affi-Gel 15 (Bio-Rad, Richmond, CA). Antibodies to the β-galactosidase component of the antigen were eliminated by absorption on an Affi-Gel 15/β-galactosidase affinity column. Anti-peptide antibodies were prepared by injecting the peptide QMNQIQSVEV (corresponding to the 10 carboxy-terminal amino acids of Cek5) coupled to BSA using glutaraldehyde. Material sufficient for five injections was prepared as follows: 5 mg of BSA dissolved in 0.5 ml of 0.4 M phosphate-buffered saline (PBS, pH 7.5) was mixed with 4 mg of peptide dissolved in H_2O; 1 ml of 2% gluteraldehyde was then added dropwise while stirring. The reaction was stopped after 30 min with 0.2 ml of 1 M glycine, and the solution was dialyzed in PBS. Injections were at 2-wk intervals and high-affinity antibodies were obtained after the fourth injection. Specific anti-peptide antibodies were purified from the immune serum by elution with 0.1 M HCl-glycine pH 2.5 from an immunoblot of 10-d embryonic brain.

Tissue preparation and immunoblotting

Whole embryo extracts, embryonic tissue extracts, and some adult tissue extracts were prepared by sonication in an ice-cold hypotonic buffer containing 1 mM $NaHCO_3$, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitor units aprotinin/ml, 10 μg/ml pepstatin, 10 μg/ml

leupeptin, and 1 mM sodium orthovanadate. Adult thigh, gizzard, intestine, and heart were homogenized in the same buffer using a tissue homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). Tissues were frozen on dry ice immediately after dissection from the embryos or from 2-y-old chickens. Tissue homogenates were stored in aliquots at −70°C. Ten-day embryonic brain was separated into nuclear, membrane, and cytosolic fractions as follows. Brain tissue was homogenized in a Dounce homogenizer in the hypotonic buffer described above. One-fifth volume of a 1.6 M sucrose, 25 mM $MgCl_2$ solution was then added. The homogenized material was centrifuged successively at 1 000, 17 000, and 200 000 × *g* to obtain the various fractions (see Results and Figure 3). For SDS-polyacrylamide gel electrophoresis, the samples were solubilized in SDS-sample buffer and heated at 95°C for 3 min. The protein concentration of the tissue homogenates was estimated by staining sample gels using 1% Coomassie blue in 25% isopropanol and 10% acetic acid. Equal amounts of protein (~150 μg) were loaded in all lanes for the immunoblotting experiments. The proteins were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose as described (Maher and Pasquale, 1988). The nitrocellulose filters were stained with amido black to confirm that equal amounts of protein were present in each lane. Filters were blocked overnight with 0.5% BSA in washing buffer (0.1% Triton X-100 in tris(hydroxymethyl)aminomethane buffered saline) and incubated for 2–4 h in washing buffer containing 3 μg/ml anti-Cek5 antibodies, 0.5% BSA, and 20 mM phosphotyrosine. After thorough rinsing with washing buffer, the immunoblots were incubated for 1 h with ^{125}I-protein A (0.25 μCi/ml, ICN, Irvine, CA) in washing buffer containing 3% BSA, rinsed thoroughly with washing buffer, and dried. The filters were autoradiographed at −70°C using Kodak (Rochester, NY) BB film and lightning plus intensifying screens. The molecular mass standards used were myosin (200 kDa), β-galactosidase (120 kDa), α-actinin (100 kDa), BSA (68 kDa), and actin (43 kDa).

Immunoprecipitation and in vitro phosphorylation

One 11-d embryonic brain was sonicated in 1.1 ml PBS containing protease inhibitors and Na orthovanadate (see above). For each immunoprecipitation, a 50-μl aliquot of the homogenate was used and diluted in 450 μl of RIPA buffer containing protease inhibitors and Na orthovanadate. Each aliquot was incubated on ice with 20 μl Staph A (Boehringer Mannheim) for 10 min and, after removal of the Staph A by centrifugation, with 15 μg of anti-Cek5 anti-fusion protein antibodies or 15 μg of control rabbit IgG preabsorbed to 20 μl Staph A, for 30 min. The immunoprecipitated material was then washed with RIPA buffer and incubated for 30 min at 30°C in a phosphorylation buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM Na orthovanadate, 0.1% Triton X-100, and 150 μM ATP. Sample buffer was then added, and the immunoprecipitates were examined by gel electrophoresis.

Nucleotide sequence accession number

The Cek5 sequence reported here has been deposited in the GenBank database under accession number M62325.

Note added in proof. After this paper was submitted for publication the complete sequence of Elk appeared in print (Lhotak *et al.*, Mol. Cell. Biol. *11,* 2496–2502).

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