

# Tissue-dependent Regulation of Protein Tyrosine Kinase Activity during Embryonic Development

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**Abstract.** Protein tyrosine kinase activity was assayed in a variety of chicken tissues during embryonic development and in the adult. In some tissues protein tyrosine kinase activity decreased during embryonic development; however, in other tissues it remained high throughout development, in contrast to the level of protein tyrosine phosphorylation, which decreased during development. The highest levels of tyrosine kinase activity were detected in 17-d embryonic brain although only low levels of protein tyrosine phosphorylation were observed in this tissue. Several alternatives

were examined in an effort to determine the mechanism responsible for the low levels of tyrosine phosphorylated proteins in most older embryonic and adult chicken tissues despite the presence of highly active tyrosine kinases. The results show that the regulation of protein tyrosine phosphorylation during embryonic development is complex and varies from tissue to tissue. Furthermore, the results suggest that protein tyrosine phosphatases play an important role in regulating the level of phosphotyrosine in proteins of many older embryonic and adult tissues.

**I**N a recent study (Maher and Pasquale, 1988), we examined the phosphorylation of proteins on tyrosine during chicken embryonic development. Proteins phosphorylated on tyrosine were found in all the embryonic tissues examined including heart, gizzard, thigh, intestine, lung, brain, liver, and kidney. However, the overall level of protein tyrosine phosphorylation in all the tissues decreased during embryonic development, and was very low or undetectable in the adult tissues. A number of mechanisms could be responsible for the decrease in the extent of protein tyrosine phosphorylation observed late in embryonic development and in the adult. First, protein tyrosine kinase activity could be very low or absent in adult tissues. Low levels of tyrosine kinase activity could be brought about by a decrease in enzyme synthesis, a posttranslational modification which results in a decrease in enzyme-specific activity or the synthesis of a specific tyrosine kinase inhibitor. However, high levels of protein tyrosine kinase activity have been detected in adult tissues from several mammalian species (Swarup et al., 1983; Okada and Nakagawa, 1988). Alternatively, the level of protein tyrosine kinase activity could be similar in embryonic and adult tissues and the absence of detectable protein tyrosine phosphorylation in adult tissues could be accounted for by an increase in protein tyrosine phosphatase activity, so that protein substrates would remain phosphorylated on tyrosine only briefly. A third possibility, also consistent with significant protein tyrosine kinase activity in adult tissues, would involve a change in the distribution of the protein tyrosine kinases or of their substrates, such that their interaction would be less favorable.

In an attempt to distinguish between these alternatives, we assayed protein tyrosine kinase activity in embryonic and adult tissues under a variety of conditions using both endogenous proteins and synthetic random amino acid copolymers as substrates. The results indicate that the regulation of protein tyrosine phosphorylation during embryonic development is complex and varies from tissue to tissue. In some tissues, such as thigh and liver, protein tyrosine kinase activity decreases during development, in parallel with the decrease in protein tyrosine phosphorylation. In other tissues, such as heart, lung, and kidney, protein tyrosine kinase activity decreases somewhat during embryonic development, but is still relatively high in the adult. Protein tyrosine phosphorylation also decreases only slightly during lung and kidney development and only falls to very low levels in the adult. In gizzard and intestine, the level of protein tyrosine kinase activity does not decrease significantly during embryonic development, but is very low in the adult. In brain, protein tyrosine kinase activity is high throughout development and in the adult, and reaches maximal levels at 17 d of development in ovo. In some of these tissues, protein tyrosine phosphatases appear to play an important role in regulating protein tyrosine phosphorylation, explaining, at least in part, the observed decrease in the overall level of protein tyrosine phosphorylation. In addition, in some tissues phosphatases appear to regulate tyrosine kinase activity. Furthermore, changes in the intracellular localization of protein tyrosine kinases in late embryonic and adult tissues were also observed. These results, which show that protein tyrosine kinase activity is modulated during embryonic development,

further suggest important roles for protein tyrosine kinases in the regulation of growth and differentiation. In addition, the finding that the overall level of protein tyrosine phosphorylation in many tissues in the adult is regulated by factors other than protein tyrosine kinase activity suggests that the consequences of protein tyrosine phosphorylation are such that it must be tightly controlled in the adult.

## Materials and Methods

### Tissue Preparation

Embryonic and adult chicken tissues were prepared as described previously (Maher and Pasquale, 1988). Briefly, tissues were removed from chicken embryos or from anesthetized 1-yr-old chickens and immediately immersed in ice-cold 1 mM NaHCO<sub>3</sub> containing 5 mM EDTA, 1 mM PMSF (Sigma Chemical Co., St. Louis, MO), 0.2 trypsin inhibitor units aprotinin/ml (Sigma Chemical Co.), 10 μM leupeptin (Sigma Chemical Co.) and 1 mM sodium orthovanadate. After thorough rinsing, the tissues were sonicated (embryonic tissues) at 4°C or finely minced (adult tissues) and then sonicated at 4°C to obtain a uniform dispersion. Aliquots were removed for protein determination by the BCA method (Pierce Chemical Co., Rockford, IL) and the remainder was stored in small aliquots at -70°C.

### Protein Tyrosine Kinase Assay

The standard tyrosine kinase assay used 25 μg protein in a total volume of 50 μl containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 50 μM sodium orthovanadate, 50 μM ATP (Sigma Chemical Co.), 2 μCi γ-(<sup>32</sup>P)ATP (10 mCi/ml; Amersham Corp., Arlington Heights, IL) and 1 mg/ml poly (Glu:Tyr; 4:1) (*M<sub>w</sub>* 45,700; Sigma Chemical Co.). The assay was carried out for 5–20 min at 30°C and stopped by the addition of 15 μl 5× sample buffer and boiling for 3 min. 30 μl of each reaction was analyzed on a 10% SDS-polyacrylamide gel prepared as described (Laemmli, 1970). The gels were stained with 1% Coomassie blue in 25% isopropanol, 10% acetic acid, destained in 10% methanol, 10% acetic acid, washed in water, dried, and autoradiographed for 15 h at -70°C. To quantify the amount of <sup>32</sup>P incorporated into the poly amino acid substrate, the sections of each lane containing the radiophosphorylated substrate were cut out and counted in a β counter (Cerenkov counts). Control reactions containing the tissue extracts but no poly amino acid substrate were run in parallel and the counts from these lanes subtracted from those containing the substrate. Although the poly amino acid substrate can only be phosphorylated on tyrosine, endogenous tissue proteins can be phosphorylated on tyrosine, serine and threonine and therefore these phosphorylations can interfere with an accurate estimate of tyrosine kinase activity. By analyzing the phosphorylation of the poly amino acid substrate by SDS-PAGE, the poly amino acid substrate can be separated away from many of the endogenous phosphoproteins. Those endogenous phosphoproteins which migrate in the same molecular weight range as the substrate can be accounted for by subtracting out the counts in the controls.

To assay for the amount of γ-(<sup>32</sup>P)ATP remaining at the end of the tyrosine kinase assays, the standard reaction was stopped by the addition of 100 μl ethanol. After precipitation at -20°C, the samples were centrifuged and the supernatants dried under vacuum. The pellets were resuspended in 10 μl 10 mM ATP and the ATP analyzed by chromatography on polyethyleneimine-cellulose plates (EM Sciences, West Germany) using 1 M LiCl. The cold ATP was located under UV light and the amount of radioactive ATP remaining determined by autoradiography.

To assay for tyrosine kinase activity using endogenous substrates, the reaction was carried out as described above except 50 μg of protein was used and no poly amino acid substrate or radioactive ATP was included. The reaction mixture was analyzed on 7.5% SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies and <sup>125</sup>I-protein A as described previously (Maher and Pasquale, 1988).

### Protein-Tyrosine Phosphatase Assay

Tyrosine phosphatase activity was assayed by a combination of the methods of Tonks et al. (1988) and Leis and Kaplan (1982). The standard assay contained 50 μg of tissue protein in a total volume of 50 μl containing 25 mM imidazole HCl, pH 7.2, 0.1% β-mercaptoethanol and 10 mM phosphotyrosine (Sigma Chemical Co.) as substrate. The reaction was carried out for 10 min at 30°C and terminated by the addition of 50 μl of 10 mg/ml BSA (United States Biochemical Corp., Cleveland, OH) and 150 μl of 25% TCA. The samples were vortexed, incubated for 10 min on ice and the precipitate was collected at 14,000 g for 5 min. The supernatants were assayed for inorganic phosphate as described (Chen et al., 1956). Control reactions were performed in the absence of tissue protein and the values subtracted from those obtained in the presence of tissue protein. The reaction was linear for 30 min at up to 80 μg tissue protein. For all the tissues examined, the phosphatase activity measured by this assay was inhibited >90% by 200 μM sodium orthovanadate.

Phase partitioning was carried out by the previously described modification (Maher and Singer, 1985) of the method of Bordier (1981). Briefly, tissue extracts were solubilized at 2 mg/ml in 1% Triton X-114 (Calbiochem-Behring Corp., La Jolla, CA) at 4°C. Insoluble material was removed by centrifugation for 5 min in a microfuge at 4°C. 300 μl of the soluble material was partitioned and the detergent phase was made equal in volume to the aqueous phase. 20 μl aliquots of the aqueous phase and the detergent phase were assayed for tyrosine kinase activity as described above.

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### Cell Fractionation

All procedures were carried out at 4°C. The tissues were disrupted in 0.32 M sucrose containing 1 mM sodium phosphate, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM PMSF, 0.2 trypsin inhibitor units aprotinin/ml, 10 μM leupeptin, and 1 mM sodium orthovanadate as described previously (deSilva et al., 1979; Ellis et al., 1988). The same method of disruption was used for all ages of the same tissue. For soft tissues this was a Dounce homogenizer and for firmer tissues, sonication or a Polytron homogenizer. The homogenate was centrifuged at 1,000 g for 5 min to yield a pellet enriched in nuclei. The supernatant was centrifuged at 17,500 g to give a pellet enriched in mitochondria and this supernatant was centrifuged at 100,000 g for 45 min to yield a microsomal pellet and a soluble fraction. The protein concentration of each fraction was determined by the BCA method and 25 μg protein from each fraction were assayed for tyrosine kinase activity as described above.

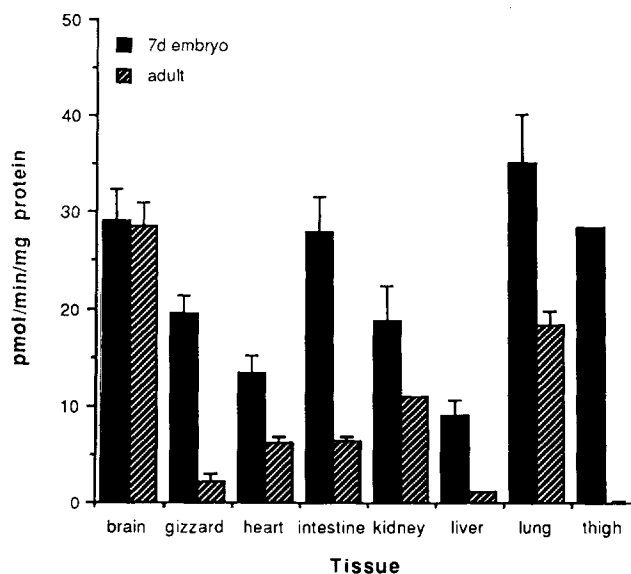
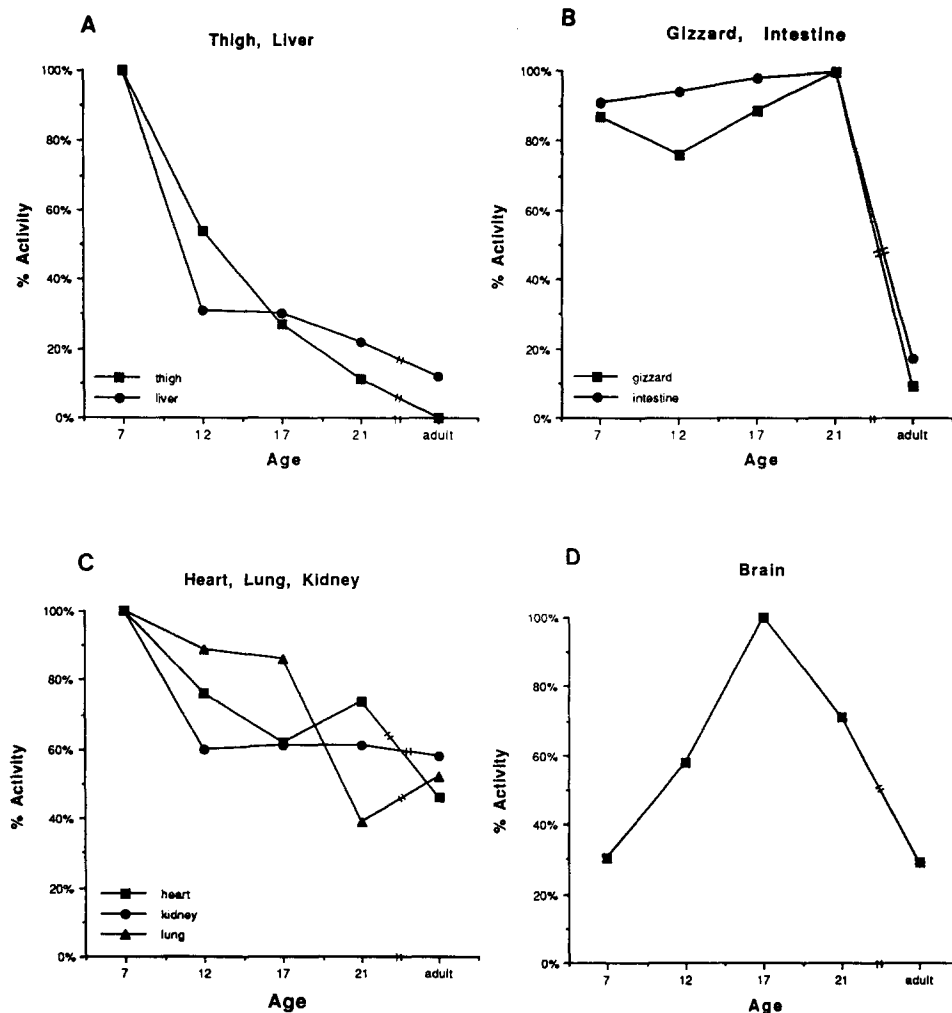


Figure 1. Protein tyrosine kinase activity in 7-d embryonic and adult tissues. Tissues were prepared and assayed for protein tyrosine kinase activity as described in Materials and Methods. The data are plotted as the specific activity of the tyrosine kinases in picomoles/minute/milligram protein. Results are the average of three to five determinations.



**Figure 2.** Protein tyrosine kinase activity as a function of embryonic development. Protein tyrosine kinase activity was assayed as described in Materials and Methods. The results are presented as the percent of the maximal tyrosine kinase activity determined in each tissue. Results are the average of three to five determinations.

## Results

The assay used to determine tyrosine kinase activity is a modification of that described by Zick et al. (1985) and contains 50 mM Tris at pH 7.4, 10 mM  $Mg^{2+}$ , 10 mM  $Mn^{2+}$ , 50  $\mu$ M vanadate to inhibit protein tyrosine phosphatases, cold ATP,  $^{32}P$ - $\gamma$ -ATP and poly (Glu, Tyr; 4:1). Maximal tyrosine kinase activity is obtained with 10–25  $\mu$ g of tissue protein per assay in a volume of 50  $\mu$ l. With higher tissue protein concentrations a decrease in tyrosine kinase activity was observed. A substrate concentration of 1 mg/ml is used since this concentration was determined to be saturating. For all tissues except lung and liver the tyrosine kinase activity remained linear for 20 min at 30°C. All ages of liver and adult lung contained significant levels of ATPase activity which resulted in a complete loss of radiolabeled ATP during a 20-min assay. However, by using a 5 min assay time for liver and a 10 min assay time for lung radiolabeled ATP could still be detected at the end of the reaction.

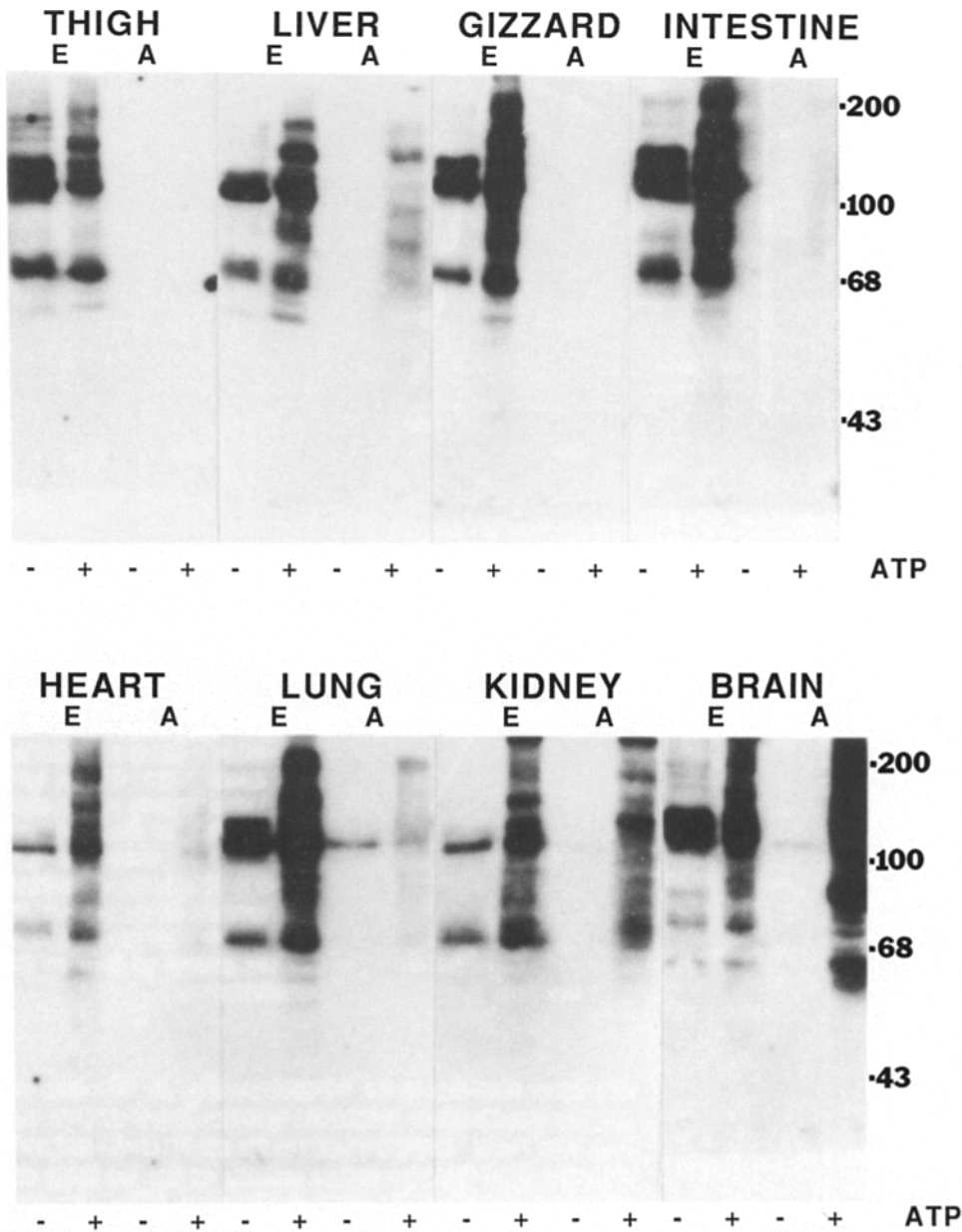
### *Tissue-dependent Changes in Protein Tyrosine Kinase Activity during Development*

When the tyrosine kinase activity in 7-d embryonic tissues was compared with that in the same tissues taken from adult chickens, the tyrosine kinase activity in the adult was gener-

ally lower than that in the 7-d embryo (Fig. 1). The only exception was the brain, where the tyrosine kinase activity was the same in the 7-d embryo and in the adult. At the opposite extreme, high tyrosine kinase activity in the 7-d thigh decreased to undetectable levels in the adult. Thus, the ratio of protein tyrosine kinase activity between the 7-d embryo and the adult varies significantly among the different tissues.

To further investigate the changes in protein tyrosine kinase activity during development, each tissue was assayed at five different stages. The results are presented in Fig. 2, plotted as the per cent of maximum activity observed in the tissue. There are four different patterns of change in tyrosine kinase activity. In the case of thigh and liver the tyrosine kinase activity decreases during development, and is very low or absent in the adult. In gizzard and intestine, the tyrosine kinase activity remains relatively constant during embryonic development but is very low in the adult. Tyrosine kinase activity in heart, lung, and kidney decreases somewhat during development but is still relatively high, as compared with the 7-d embryo in the adult. The brain is unique among the tissues examined in that the tyrosine kinase activity is maximal in the 17-d embryonic brain. At this time the average specific activity is 97 pmol/min per mg protein, the highest level seen in any tissue at any time of development.

One simple explanation for the decreased tyrosine kinase



*Figure 3.* Immunoblotting, with rabbit anti-phosphotyrosine and  $^{125}\text{I}$ -protein A, of 50  $\mu\text{g}$  samples of 10-d embryonic and adult tissues incubated in kinase buffer in absence (-) or presence (+) of ATP for 20 min at 30°C before analysis by SDS-PAGE. *E*, tissue extracts from 7-d embryonic chickens; *A*, tissue extracts from adult chickens.

activities observed in adult tissues is that poly (Glu, Tyr; 4:1) is not a very good substrate for the tyrosine kinases in adult tissues. However, the lower tyrosine kinase activities observed in most of the adult tissues does not seem to reflect a major change in substrate specificity occurring during development. In fact, results similar to those obtained with poly (Glu, Tyr; 4:1) were also obtained using two other synthetic tyrosine kinase substrates, poly (Glu, Ala, Tyr; 6:3:1; Braun et al., 1984; Zick et al., 1985) and poly (Glu, Tyr; 1:1; Yonezawa and Roth, 1990) (not shown) as well as endogenous substrates (Fig. 3). To study the phosphorylation on tyrosine of endogenous substrates, the tyrosine kinase activities in embryonic and adult tissues were compared by performing the phosphorylation assay in the absence (-) and presence (+) of cold ATP alone and analyzing the phosphorylation of the substrates by immunoblotting with anti-phosphotyrosine antibodies. The phosphorylation of all the endogenous substrates in both the embryonic and adult tissue

extracts could be competitively inhibited by including excess poly (Glu, Tyr; 4:1) in the reaction mixture further suggesting that substrate specificity does not change significantly during development. The lower levels of tyrosine kinase activity detected in some adult tissues (e.g., heart, lung) by this assay as compared with the assay using radiolabeled ATP and poly (Glu, Tyr; 4:1) probably reflect the difference in detection techniques used.

Developmental changes in the ion requirements of the tyrosine kinases also do not appear to be involved in the lower levels of tyrosine kinase activity observed in many older embryonic and adult tissues. Tyrosine kinase activity was assayed in embryonic and adult tissues in the absence of divalent cations, in the presence of  $\text{Mg}^{2+}$  alone, in the presence of  $\text{Mn}^{2+}$  alone and in the presence of both divalent cations. In all cases, the maximal tyrosine kinase activity was obtained in the presence of both divalent cations (not shown).

**Table I. Tyrosine Phosphatase Activity in Embryonic and Adult Tissues**

Tissue	Phosphatase activity nmol/min/mg protein
7-d brain	19.2 ± 2.1
10-d brain	22.9 ± 3.4
19-d brain	32.2 ± 4.2
Adult brain	23.7 ± 1.0
10-d gizzard	14.0 ± 1.6
19-d gizzard	12.5 ± 1.3
10-d heart	6.8 ± 2.6
Adult heart	5.2 ± 0.5
10-d intestine	13.8 ± 1.8
19-d intestine	37.7 ± 7.5
10-d kidney	20.8 ± 1.8
Adult kidney	38.2 ± 8.1
10-d liver	21.8 ± 1.8
19-d liver	37.2 ± 4.7
10-d lung	7.8 ± 2.6
19-d lung	6.5 ± 0.8
7-d thigh	8.4 ± 2.5
19-d thigh	6.5 ± 1.6

Tissue homogenates were prepared in the absence of sodium orthovanadate and assayed for tyrosine phosphatase activity as described in Materials and Methods. The results are the average of three to four separate experiments run in duplicate.

#### **Changes in Protein Tyrosine Phosphatase Activity during Embryonic Development**

Although in most tissues protein tyrosine kinase activity was lower in the late embryos and adult than in the 7-10 d embryo, the significant levels of tyrosine kinase activity present in many of the tissues of the late embryo and adult were not consistent with the extremely low levels of tyrosine phosphorylated proteins observed by immunoblotting these same tissues with anti-phosphotyrosine antibodies (Maher and Pasquale, 1988). For example, 19-d gizzard and intestine have levels of tyrosine kinase activity equal to or greater than those observed in the same tissues taken from 10-d embryos, but only barely detectable amounts of tyrosine phosphorylated proteins. An increase in protein tyrosine phosphatase activity during development could explain, at least in part, this discrepancy. This possibility was tested by preparing tissue extracts in the absence of sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, and assaying for phosphotyrosine phosphatase activity. As shown in Table I, the level of phosphatase activity varied greatly between tissues. In all cases, the phosphatase activity was inhibited >90% by 200 μM sodium orthovanadate. Another putative phosphatase inhibitor, phenylarsine oxide (Bernier et al., 1988; Fallon, 1990), had no effect on phosphatase activity. In some tissues, such as liver, kidney, intestine, and brain, the phosphatase activity increased significantly later in development. Thus, an increase in protein tyrosine phosphatase activity may regulate the level of protein tyrosine phosphorylation in some tissues late in embryonic development and in the adult.

Although phosphotyrosine phosphatases will degrade the products of tyrosine kinase activity, they are also known to directly activate (e.g., Mustelin et al., 1989; Tonks and

Charbonneau, 1989) and inhibit (Lin and Clinton, 1988; Swarup and Subrahmanyam, 1989) tyrosine kinases. To obtain evidence for a tissue-dependent activation or inhibition of tyrosine kinases by phosphatases, tissue extracts were prepared in the absence of vanadate, to allow endogenous phosphatases to dephosphorylate any tyrosine kinases in the extract, and assayed for tyrosine kinase activity in the presence of vanadate, to prevent substrate dephosphorylation. The data were compared to those obtained from extracts prepared in the presence of vanadate and assayed simultaneously. The results of this comparison are shown in Table II. Most of the early embryonic tissues appear to contain tyrosine kinases that can be activated by dephosphorylation since the tyrosine kinase activity is greater in the tissue extracts prepared in the absence of vanadate. The exception is 10-d brain where tyrosine kinase activity is lower in the absence of vanadate. In contrast, the tyrosine kinase activity in most of the older embryonic and adult tissues is unaffected or decreased in the extracts prepared in the absence of vanadate. The exceptions are adult kidney and brain where tyrosine kinase activity is higher in the absence of vanadate.

#### **Changes in the Distribution of Tyrosine Kinases during Embryonic Development**

A second explanation for high levels of tyrosine kinase activity but low levels of protein tyrosine phosphorylation could be that although both the tyrosine kinases and the substrates may be present, the substrates may no longer be accessible to the kinases. Evidence indicating that changes in the intracellular localization of the tyrosine kinases occurs during development was obtained by comparing the intracellular dis-

**Table II. Effect of Vanadate on Protein Tyrosine Kinase Activity**

Tissue	Ratio activity +VO <sub>3</sub> /-VO <sub>3</sub>
7-d brain	0.48 ± 0.02
10-d brain	2.37 ± 0.10
19-d brain	0.86 ± 0.15
Adult brain	0.36 ± 0.10
10-d gizzard	0.62 ± 0.11
19-d gizzard	1.11 ± 0.14
10-d heart	0.57 ± 0.11
Adult heart	2.61 ± 0.45
10-d intestine	0.49 ± 0.08
19-d intestine	1.22 ± 0.32
10-d kidney	0.56 ± 0.01
Adult kidney	0.56 ± 0.04
10-d liver	0.60 ± 0.06
19-d liver	1.74 ± 0.46
10-d lung	1.19 ± 0.06
19-d lung	0.92 ± 0.04
7-d thigh	0.53 ± 0.03
19-d thigh	1.48 ± 0.07

Tissue homogenates were prepared in the absence and presence of 1 mM sodium orthovanadate and assayed for protein tyrosine kinase activity using poly (Glu, Tyr; 4:1) in the presence of sodium orthovanadate as described in Materials and Methods. Results are presented as the ratio of the tyrosine kinase activity in homogenates prepared in the presence of sodium orthovanadate to the tyrosine kinase activity in homogenates prepared in the absence of sodium orthovanadate and are the average of three separate experiments.

**Table III. Protein Tyrosine Kinase Activity in Tissues Fractionated by Phase Partition with Triton X-114**

Tissue	Aqueous	Detergent
	% total protein tyrosine kinase activity	
7-d brain	39.5 ± 1.5	60.5 ± 1.5
10-d brain	37.7 ± 3.5	62.3 ± 3.5
19-d brain	61.3 ± 5.8	38.7 ± 5.8
Adult brain	63.0 ± 6.0	37.0 ± 6.0
10-d gizzard	36.0 ± 4.0	64.0 ± 4.0
19-d gizzard	80.0 ± 3.0	20.0 ± 3.0
10-d heart	44.3 ± 4.1	55.7 ± 4.1
Adult heart	65.0 ± 7.0	35.0 ± 7.0
10-d intestine	35.0 ± 1.0	65.0 ± 1.0
19-d intestine	67.5 ± 3.5	32.5 ± 3.5
10-d kidney	59.0 ± 3.7	41.0 ± 3.7
Adult kidney	71.5 ± 0.5	28.5 ± 0.5
10-d liver	50.0 ± 1.0	50.0 ± 1.0
19-d liver	53.7 ± 3.3	46.3 ± 3.3
10-d lung	53.5 ± 2.5	46.5 ± 2.5
19-d lung	72.0 ± 3.0	28.0 ± 3.0
7-d thigh	63.0 ± 1.0	37.0 ± 1.0
19-d thigh	77.0 ± 6.0	23.0 ± 6.0

Tissue homogenates were fractionated by phase partition in Triton X-114 into aqueous and detergent phases as described in Materials and Methods. The protein tyrosine kinase activity in 10- $\mu$ l aliquots of the two phases was determined as described in Materials and Methods. The total protein tyrosine kinase activity was calculated by summing the tyrosine kinase activity in the two phases. The results are the average of three separate experiments.

tribution of tyrosine kinase activity between early embryonic and late embryonic or adult tissues. Tissues were fractionated in two ways. In the first instance, phase partition in the detergent Triton X-114 was used to separate tissues into integral membrane and membrane-associated proteins (detergent phase) and soluble proteins (aqueous phase). The results are shown in Table III. In many of the early embryonic tissues examined, including brain, heart, gizzard, and intestine, the majority of the tyrosine kinase activity was found in the detergent fraction containing integral membrane or membrane-associated proteins. However, later in embryonic development and in the adult, these tissues showed a shift in the distribution of kinase activity such that the majority of the tyrosine kinase activity was now found in the aqueous fraction which contains soluble proteins. Liver and thigh showed little or no change in the distribution of tyrosine kinase activity and lung and kidney only showed moderate changes.

The second fractionation method relied on the technique of differential centrifugation, such that a tissue homogenate was separated into nuclear, mitochondrial, microsomal and soluble fractions. For a given tissue, the same homogenization technique was used for both the early embryo and the late embryo or adult sample so that the fractionation results would not be simply a reflection of the more stringent homogenization required by many older tissues as compared with their embryonic counterparts. The results of these fractionations are shown in Fig. 4. In all the tissues examined a change in the distribution of tyrosine kinase activity was seen during embryonic development. Gizzard, liver, thigh, kidney, and adult brain showed particularly large increases in the relative amount of tyrosine kinase activity found in the

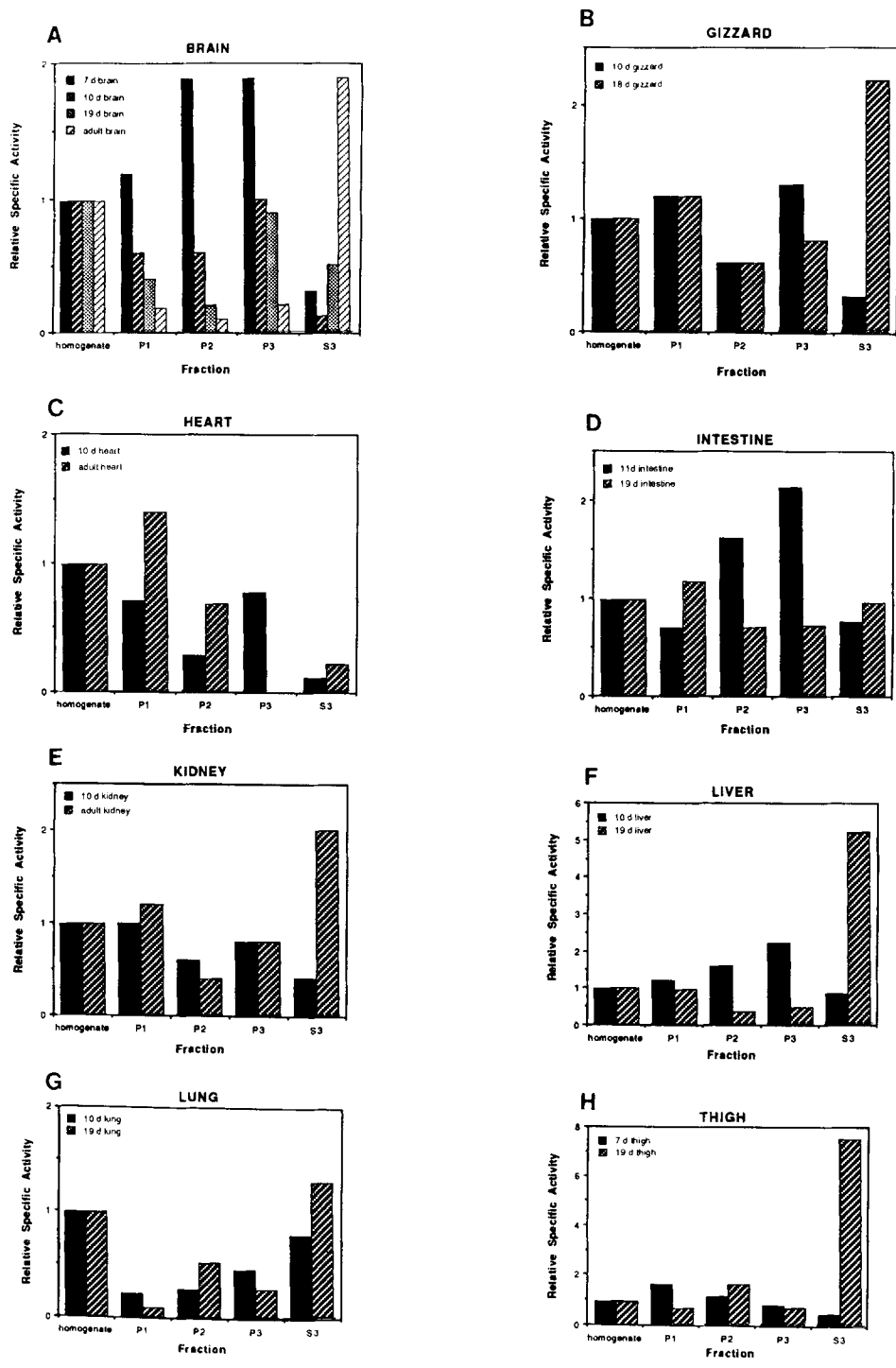
soluble fraction. In agreement with the phase partition results, the relative amount of tyrosine kinase activity associated with the cell membrane fraction decreased during the development of most tissues. The level of phosphotyrosine-containing proteins in the different fractions during development was not compared because the level is very low or undetectable in the late embryonic and adult tissues. However, in the 7-10 d embryonic tissues, proteins phosphorylated on tyrosine were not enriched in fractions containing high levels of tyrosine kinase activity.

## Discussion

In this study the mechanisms that regulate protein tyrosine phosphorylation during embryonic development in the chicken were examined. We had previously observed that although proteins phosphorylated on tyrosine were readily detectable in embryonic tissues, particularly between 7 and 15 d of development in ovo, they were barely detectable in older embryonic tissues (18-21 d in ovo) and were generally absent in the adult. One simple explanation for the decrease in protein tyrosine phosphorylation during embryonic development is that tyrosine kinase activity also decreases at the same time. However, in many tissues this is not the correct explanation. The only tissues where tyrosine kinase activity changed in a pattern similar to that observed for protein tyrosine phosphorylation were thigh and liver. In kidney and lung both tyrosine kinase activity and protein tyrosine phosphorylation decreased slightly during embryonic development but while tyrosine kinase remained relatively high in these tissues from the adult, protein tyrosine phosphorylation was very low. Although most of the other tissues examined showed changes in tyrosine kinase activity during development, these changes were not sufficient to account for the large decreases observed in protein tyrosine phosphorylation.

There are a number of mechanisms that could account for the decrease in protein tyrosine kinase activity observed in at least some tissues during development. First, the synthesis of tyrosine kinases could be lower. There are several precedents for the decrease in the synthesis of a tyrosine kinase during development. In cultured muscle cells the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) receptors disappear, as assayed by ligand binding, concomitant with differentiation (Olwin and Hauschka, 1988). Both the bFGF receptor and the EGF receptor are tyrosine kinases. Furthermore, although a bFGF receptor is present in most tissues taken from the 10-d chick embryo, it is only detectable in the adult chicken brain (Pasquale and Singer, 1989). In rats, there is evidence for a distinct fetal IGF I-related receptor in muscle which is not present in adults (Alexandrides and Smith, 1989).

A second possibility is that tyrosine kinases are still present in the late embryonic and adult tissues, but are inhibited or no longer activated. Inhibition of a tyrosine kinase can be caused by either a posttranscriptional modification or by the binding of an inhibitor. The tyrosine kinase pp60<sup>src</sup>, for example, is subject to both types of modification. Phosphorylation of pp60<sup>src</sup> at tyrosine 527 causes a decrease in tyrosine kinase activity (Cooper and King, 1986) and the interaction of pp60<sup>src</sup> with two proteins, pp90 and pp50, appears to block substrate phosphorylation (Brugge, 1986). However, a decrease in activity induced by tyrosine phosphorylation



**Figure 4.** Specific activity (picoles/minute/milligram protein) of tyrosine kinases in tissue fractions from early embryonic, late embryonic and adult chickens relative to the specific activity (picoles/minute/milligram protein) of tyrosine kinases in the whole tissue homogenate. Tissues were fractionated as described in Materials and Methods and 25  $\mu$ g protein from each fraction was assayed for protein tyrosine kinase activity. Results are the average of two to three determinations.

does not appear to be important in most older embryonic and adult chicken tissues, except kidney and brain, since preincubation of tissue extracts in the absence of vanadate did not increase the tyrosine kinase activity observed in the older tissues. Preincubation in the absence of vanadate did increase the tyrosine kinase activity in most of the younger embryonic tissues suggesting that negative regulation of tyrosine kinases by phosphorylation may be important earlier in development. The presence of a tyrosine kinase inhibitor later in development is suggested by the fractionation data with 19-d thigh, liver, and gizzard and adult kidney and brain. The solu-

ble fractions from all these tissues contain levels of tyrosine kinase activity comparable to those found in the same tissues taken from 7-10 d embryos, although the tissue homogenates contain very low levels of tyrosine kinase activity. Preliminary evidence for endogenous inhibitors in several of these tissues has come from mixing experiments where the addition of aliquots of 19-d thigh extract to 7-d thigh extracts leads to a significant decrease in tyrosine kinase activity. An endogenous inhibitor of tyrosine kinase activity has been reported recently in lymphoid cells (Hall et al., 1989).

A third possibility is that the kinases are still present in the

older tissues but are no longer activated. An example of a lack of activation would be a growth factor receptor that is no longer exposed to the growth factor, since most growth factor receptors exhibit lower levels of tyrosine kinase activity in the absence of the appropriate ligand. In late embryos or adults some growth factors may no longer be present or may not be accessible to their receptors. If this explanation was correct, treatment of tissue extracts from late embryonic or adult chickens with appropriate growth factors might activate tyrosine kinases. Preliminary studies with thigh extracts and a variety of growth factors have shown no stimulation of tyrosine kinase activity. However, evidence for a decrease in tyrosine kinase activity induced by tyrosine dephosphorylation in several of the older embryonic and adult tissues is provided by the data shown in Table II. In contrast to their early embryonic counterparts, preparation of adult heart and 19-d liver and thigh in a buffer lacking vanadate before the tyrosine kinase assay leads to a significant decrease in tyrosine kinase activity as compared with samples prepared in the presence of vanadate.

Another question is how can the relatively high levels of tyrosine kinase activity in many late embryonic or adult tissues be reconciled with the low levels of protein tyrosine phosphorylation seen in these tissues? In several tissues, protein tyrosine phosphatase activity appears to increase during development. This is especially evident in the late embryonic brain where the overall level of tyrosine kinase activity is extremely high but little protein tyrosine phosphorylation is detectable. 19-d intestine and adult kidney also exhibit high levels of protein tyrosine phosphatase activity relative to their 10-d embryonic counterparts, consistent with moderate levels of tyrosine kinase activity and little or no detectable protein tyrosine phosphorylation. Thus, these tissues provide evidence that protein tyrosine phosphatases can play a critical role in regulating the overall level of protein tyrosine phosphorylation in cells *in vivo*.

Two families of protein tyrosine phosphatases have recently been described (for reviews see Tonks and Charbonneau, 1989; and Hunter, 1989). The low molecular weight phosphatases were originally isolated as 37-kD soluble proteins (Tonks et al., 1988) but cDNA cloning suggests that these are truncated forms of a ~50-kD protein (Cool et al., 1989). The additional ~11 kD has been proposed to have two functions; it may localize the phosphatase to the cell membrane via posttranslational modifications and it may down-regulate, either directly or indirectly through interaction with regulatory molecules, the activity of the phosphatase. The high molecular weight phosphatases are integral membrane proteins which have the structure of cell surface receptors (Tonks and Charbonneau, 1989). The mRNA encoding a low molecular weight phosphatase is found at high levels in human adult liver and kidney whereas only low levels are found in adult heart and lung (Chernoff et al. 1990). The mRNA encoding a high molecular weight phosphatase shows a similar distribution in adult mouse tissues (Sap et al., 1990). These findings are consistent with the results presented in Table I on phosphatase activity in older embryonic and adult chicken tissues.

Another mechanism for regulating protein tyrosine phosphorylation would be to change the distribution of substrates and/or kinases so they can no longer interact. Two types of fractionation were used to determine if the distribution of

tyrosine kinase activity changed in tissues during development. The phase partition method separates proteins on the basis of affinity for detergent. Therefore, many, but not all, membrane proteins, as well as a number of membrane-associated proteins (e.g., pp60<sup>c-src</sup> [unpublished results]), are found in the detergent fraction, whereas most other cellular proteins are found in the aqueous fraction. In most tissues examined the percent of the total tyrosine kinase activity found in the detergent fraction decreased in the late embryo and adult. This decrease was most apparent in the gizzard. These results suggest that membrane-associated tyrosine kinases are not as active later in development. As discussed above, the decrease in tyrosine kinase activity in the membrane fraction could be due, at least partially, to growth factor receptors which are no longer being synthesized or being stimulated. Alternatively, it could be due to structural modifications to membrane-associated kinases such that they no longer associate with the cell membrane. The second fractionation method separates cellular organelles on the basis of density. This type of fractionation provides an indication of whether the association changes during development. The most noticeable change is a large increase in the tyrosine kinase activity associated with the soluble fraction in adult brain and kidney and 19-d gizzard, thigh and liver. In most cases the increase in the soluble tyrosine kinase activity coincides with a decrease in membrane-associated tyrosine kinase activity but may also reflect the presence of tyrosine kinase inhibitors in the other fractions.

Another explanation for a decrease in protein tyrosine phosphorylation could be the loss of some substrates. In many tissues, there appear to be only two or three major physiological substrates (Maher and Pasquale, 1988). If these are no longer synthesized by the cell then tyrosine phosphorylation would not be evident. However, when the known tyrosine kinase substrates which have been examined either during development or in adult tissues are considered, no clear pattern emerges. The amount of the tyrosine kinase substrate p36 decreases during the maturation of some chicken tissues but increases in others (Greenberg et al., 1984), whereas the tyrosine kinase substrate p81 is found in a number of adult rat tissues (Gould et al., 1986).

This study is the first systematic examination of protein tyrosine kinase activity in the embryonic and adult chicken and shows that the decreases in protein tyrosine phosphorylation seen during embryonic development of the chicken are due to a variety of different factors which are tissue dependent. In the thigh and liver, the decrease in protein tyrosine phosphorylation can be explained predominantly on the basis of a decrease in tyrosine kinase activity. 19-d brain, intestine, and adult kidney all appear to have high levels of protein tyrosine phosphatase activity. However, the presence of active protein tyrosine phosphatases and a decrease in tyrosine kinase activity cannot explain the low levels of protein tyrosine phosphorylation observed in 19-d gizzard and adult heart. Changes in the distribution or in the identity of the tyrosine kinases may function in gizzard in regulating protein tyrosine phosphorylation, but are probably not as important in adult heart. The regulation of protein tyrosine phosphorylation in this tissue may be due to other factors such as substrate loss. In addition, some of these tissues, such as 19-d thigh, liver, and gizzard and adult kidney and brain may contain endogenous inhibitors of tyrosine kinase



activity that further serve to downregulate tyrosine kinase activity.

These results indicate that the regulation of protein tyrosine phosphorylation during embryonic development is a complex process involving a variety of different mechanisms. The mechanisms involved vary from tissue to tissue and presumably reflect the properties of each tissue in adult life.

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