Tyrosine Phosphorylated Proteins in Different Tissues during Chick Embryo Development

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Abstract. A high affinity polyclonal antibody specific for phosphotyrosyl residues has been used in immunoblotting experiments to survey developing embryonic chicken tissues for the presence and characteristics of tyrosine phosphorylated proteins. Proteins phosphorylated on tyrosine were found to be present in all the embryonic tissues examined, including heart, thigh, gizzard, intestine, lung, liver, kidney, brain, and lens, from 7 to 21 d of development in ovo, but were greatly reduced or absent in the same tissues taken from adult chickens. A limited number of major tyrosine phosphorylated proteins were seen in all the tissues examined and they ranged in molecular mass from 35 to 220 kD. Most of the tissues contained proteins phosphorylated on tyrosine with apparent molecular masses of 120, 70, 60, and 35 kD, suggesting that the substrates of tyrosine protein kinases in different tissues may be related proteins. One-dimensional peptide mapping of the 120- and 70-kD protein bands indicated a close structural relationship among the phosphotyrosine-containing proteins of 120 kD, and similarly among those of 70 kD, from the different tissues.

EMBRYONIC development requires the coordinated expression of a repertoire of cellular activities including cell division and differentiation. Although the regulation of these activities is critical for normal development, the mechanisms that modulate cell division and differentiation are not yet known. A suggestion as to some of the cellular proteins involved in growth regulation has come from studies on retroviruses. These viruses carry homologues of cellular genes whose unregulated expression transforms cells to a state of rapid and uncontrolled growth and at least partial dedifferentiation (Bishop, 1985). The cellular forms of these genes (proto-oncogenes) have therefore been postulated to play critical roles in regulating cell division and differentiation. Many of the proto-oncogenes (e.g., c-src, c-erbB, c-fms) encode proteins displaying tyrosine kinase activity (for review see Hunter and Cooper, 1986). The receptors for various growth factors, including platelet-derived growth factor (PDGF)[1] (Frackelton et al., 1984; Ek and Heldin, 1984), insulin (Kasuga et al., 1982) and insulin-like growth factor I (Sasaki et al., 1985), are also protein tyrosine kinases, different from those encoded by known proto-oncogenes. These findings suggest that protein tyrosine kinases could be involved in regulating cell division and differentiation during embryonic development.

Over the past few years a variety of vertebrate embryos have been examined for the presence of protein and/or mRNA corresponding to proto-oncogenes encoding protein tyrosine kinases (for review see Adamson, 1987). Many of

the studies have focused on c-src, the cellular counterpart of the transforming gene of Rous sarcoma virus. The amount of the protein encoded by c-src was found to change during embryonic development and to vary from tissue to tissue, with the highest levels in brain and other neural tissues (Jacobs and Rubsamen, 1983; Cotton and Brugge, 1983; Schartl and Barnekow, 1984; Levy et al., 1984), suggesting that pp60[c-src] plays a role in neuronal development.

To study the tyrosine phosphorylation of proteins during embryonic development, we have used recently described high affinity polyclonal rabbit antibodies specific for phosphotyrosyl residues in proteins (Wang, 1985). These antibodies react with a wide range of proteins bearing phosphotyrosyl residues, even detecting some in normal cell lines (Maher et al., 1985; Morla and Wang, 1986; Pasquale, E. B., P. A. Maher, and S. J. Singer, manuscript submitted for publication). For the studies to be described, extracts were prepared from various tissues at different stages of development of the chick embryo and were examined for the presence of phosphotyrosine-containing proteins by immunoblotting with anti–phosphotyrosine antibodies. We found proteins phosphorylated on tyrosine in all of the embryonic tissues examined. The overall level of protein tyrosine phosphorylation in most of the tissues decreased during embryonic development falling to very low or undetectable levels in the tissues of the adult. There were fewer than ten major tyrosine phosphorylated protein bands in all the tissues examined and they ranged in molecular mass from 35 to 220 kD. In almost all the tissues, proteins with molecular masses of 120, 70, 60, and 35 kD were phosphorylated on tyrosine. The amount of phosphotyrosine in these proteins changed during develop-

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1. *Abbreviation used in this paper*: PDGF, platelet-derived growth factor.

ment, and the time course of the appearance and disappearance of phosphotyrosine-containing proteins in the different tissues was examined. These results, which show that tyrosine phosphorylation of proteins is modulated during embryonic development, further suggest important roles for protein tyrosine kinases in the regulation of cell division and differentiation. In addition, the proteins phosphorylated on tyrosine at 120 and 70 kD from several different embryonic tissues were isolated from SDS-polyacrylamide gels, subjected to limited proteolysis with V8 protease, and immunoblotted with the anti–phosphotyrosine antibodies. The results indicate a close structural relationship among the phosphotyrosine-containing proteins of 120 kD, and similarly among those of 70 kD, in the different tissues. Indirect immunofluorescence labeling experiments with the anti–phosphotyrosine antibodies were carried out on primary cultures of 8–10 d cardiac, lens, endothelial, and gizzard cells to characterize further some of the proteins phosphorylated on tyrosine. In the paper to follow (Takata and Singer, 1988), the anti–phosphotyrosine antibodies have been used in immunofluorescence and immunoelectron microscopic studies to examine the localization of some of the phosphotyrosine-modified proteins in several embryonic chick tissues.

Materials and Methods

Tissue Preparation

Tissues were removed from chicken embryos or from anesthetized 1-yr-old chickens and immediately immersed in ice-cold 1 mM NaHCO$_3$ containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (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 tissue) at 4°C or briefly homogenized in a Waring blender (adult tissue) at 4°C to obtain a uniform dispersion. Aliquots were removed for protein determination (Hartree, 1972), and the remainder was stored in small aliquots at −70°C. Rous sarcoma virus–transformed chick embryo fibroblasts were provided by B. Sefton (Salk Institute).

SDS-PAGE and Immunoblotting

Samples were solubilized in SDS-sample buffer, sonicated, boiled for 3 min, and equal volumes containing 75 μg protein applied to 7.5% SDS-polyacrylamide gels prepared as described (Laemmli, 1970). The proteins were then transferred to nitrocellulose as described (Towbin et al., 1979), stained with amido black to confirm the presence of equal amounts of protein in each lane, destained with 10% methanol, 10% acetic acid, blocked for at least 2 h in 3% BSA in Tris-buffered saline (TBS), and incubated for at least 4 h with 2 μg/ml rabbit anti–phosphotyrosine antibodies prepared as described below. After thorough washing with TBS, the immunoblots were incubated for 1 h with ^{125}I-protein A (0.25 μCi/ml; ICN, Irvine, CA) in 3% BSA in TBS, rinsed extensively in TBS and briefly in 0.2% NP-40 in 0.5% BSA in TBS, dried, and autoradiographed at −70°C using preflashed Kodak BB film and lightning plus intensifying screens. In some cases the gels were stained with 1% Coomassie Blue in 25% isopropanol, 10% acetic acid. The molecular mass standards used were filamin (250 kD), myosin (200 kD), vinculin (120 kD), α-actinin (100 kD), BSA (68 kD), and actin (43 kD).

One-dimensional Peptide Mapping

One-dimensional peptide maps of the 120- and 70-kD phosphotyrosine-containing protein bands were carried out as described (Cleveland et al., 1977) using 10 ng/well V8 protease (Sigma Chemical Co.). The two protein bands were identified by staining the gels for 15 min with 1% Coomassie Blue to visualize the molecular mass markers. The 120-kD band moved just above the vinculin molecular mass marker and the 70-kD band moved just above the BSA molecular mass marker.

Immunoprecipitation Experiments

Extracts of 8-d embryonic thigh were diluted to 1 mg/ml with RIPA buffer (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) and centrifuged at 20,000 g for 15 min. 100-μl aliquots of the supernatant were mixed either with rabbit anti-vinculin (20 μg) absorbed to Staphylococcus aureus beads (20 μl; Boehringer Mannheim), or the anti–integrin antibody mAb 30B6 (Maher and Singer, 1988) coupled to Ultrogel ACA-22, for 1 h at room temperature. The immunoprecipitated proteins were washed three times with RIPA buffer, once with 50 mM Tris-Cl, pH 7.5, and eluted with SDS-sample buffer. The extracts before and after immunoprecipitation and the immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with rabbit anti–phosphotyrosine antibodies as described above, with rabbit anti-vinculin antibodies (1 μg/ml) followed by ^{125}I-protein A or with mAb 30B6 (10 μg/ml) followed by rabbit anti-mouse (1 μg/ml) and ^{125}I-protein A. The samples for immunoblotting with mAb 30B6 were eluted with 0.2 M HCl-glycine, pH 2.8 for 1 h at room temperature, and then solubilized in SDS-sample buffer without mercaptoethanol.

Immunofluorescence Microscopy

Primary cultures of cardiac, gizzard, and lens cells were prepared by trypsinization of heart, gizzard, and lens taken from 8-d chicken embryos. The cells were cultured on coverslips in MEM containing 10% FCS at 37°C. All subsequent steps were carried out at room temperature. Cells were rinsed in PBS, fixed for 5 min with 3% formaldehyde (vol/vol) in PBS, and permeabilized for 5 min with 0.5% Triton X-100 in 10 mM Hepes, pH 7.4, 300 mM sucrose, 3 mM MgCl$_2$, 50 mM NaCl, 0.1 mM sodium orthovanadate. Permeabilized cells were treated with rabbit anti–phosphotyrosine antibodies (3 μg/ml) in PBS for 30 min, rinsed in PBS, labeled with a mixture of rhodamine-conjugated affinity purified F(ab)$_2$ fragment of goat anti-rabbit IgG (8 μg/ml; Jackson Immunoresearch, Avondale, PA) and nitrobenzoxadiazole (NBD)-phallacidin (20 U/ml; Molecular Probes, Junction City, OR) in PBS for 10 min, and mounted in 90% glycerol, 10 mM Tris-Cl, pH 8.0. Labeled cells were examined with a Zeiss Photomicroscope III using a 63× numerical aperture 1.4 Planapochromat oil objective and photographed on Kodak Tri-X film.

Antibody Preparation

To prepare anti–phosphotyrosine antibodies, the insoluble material obtained after sonication of bacteria expressing the v-abl-encoded transforming protein in its tyrosine phosphorylated form was used to immunize rabbits (Wang, 1985). The immune serum was purified on an immunoaffinity column prepared by linking O-phospho-L-tyrosine (Sigma Chemical Co.) to Affigel-15 (Biorad Laboratories, Richmond, CA). The anti–phosphotyrosine antibodies that specifically bound to the phosphotyrosine groups of the column were eluted with 40 mM phenylphosphate (Calbiochem-Behring Corp., La Jolla, Ca) in PBS. After dialysis against PBS and addition of 0.3% BSA, the antibodies were frozen in aliquots.

Protein Determination

The protein concentrations of the tissue homogenates were estimated by determining the absorbance at 280 nm of a 1:100 dilution in 1% SDS warmed to 37°C and comparing the result to a standard curve prepared using the Hartree (1972) protein assay run on several of the tissue samples.

Results

To determine whether protein tyrosine phosphorylation occurred during chicken embryonic development, embryos were taken from day 7 to day 21 in ovo, the various tissues removed, placed in a hypotonic buffer containing protease and phosphatase inhibitors, sonicated, and stored at −70°C. This procedure for sample preparation gave the most efficient solubilization of the phosphotyrosine-containing proteins in the embryonic tissues. The phosphotyrosine-containing proteins, which were prepared by direct solubilization in SDS-sample buffer, appeared to aggregate at the top of the separating gels giving weaker and less uniform immuno-
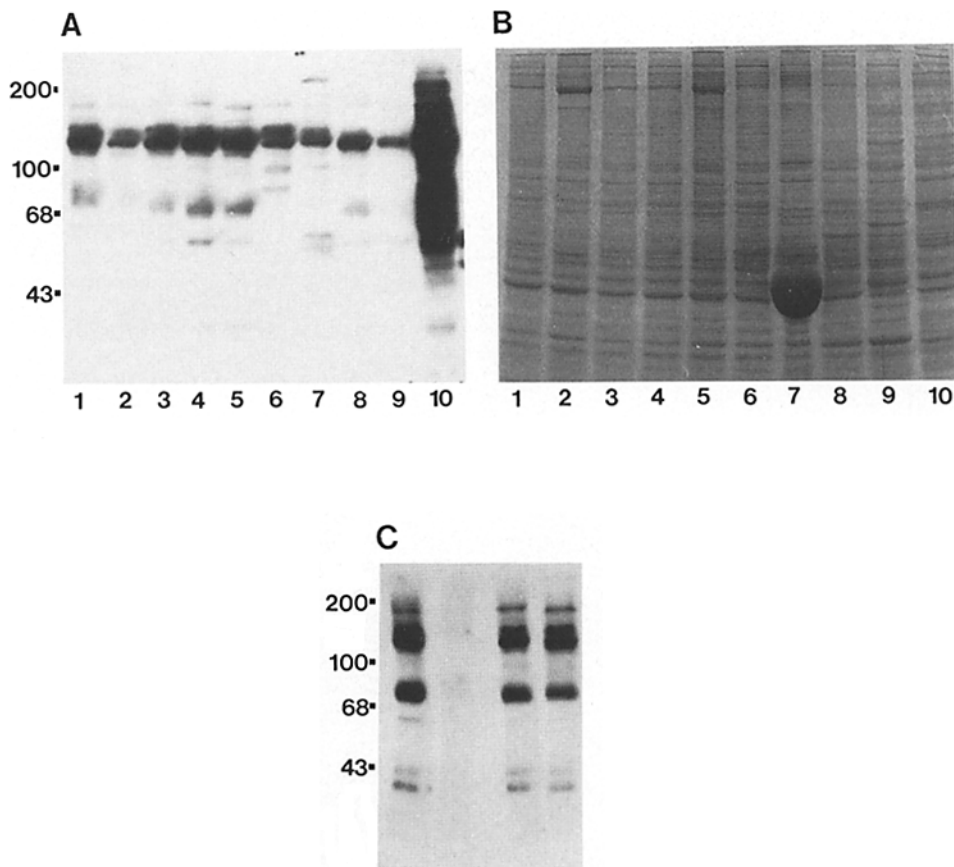
Figure 1. (A). Immunoblotting of embryonic tissue extracts with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A. Lane 1, 8-d gizzard; lane 2, 8-d heart; lane 3, 8-d thigh; lane 4, 8-d intestine; lane 5, 9-d lung; lane 6, 11-d brain; lane 7, 9-d lens; lane 8, 9-d kidney; lane 9, 9-d liver; and lane 10, Rous sarcoma virus–transformed chick embryo fibroblasts. (B) Coomassie Blue staining of a gel of embryonic tissue extracts. Lanes are the same as in Figure 1 A. (C) Immunoblotting of 8-d embryonic thigh with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A alone (lane 1) or in the presence of 10 mM phosphotyrosine (lane 2), 10 mM phosphoserine (lane 3), and 10 mM phosphothreonine (lane 4).

blots. Fig. 1 A shows a comparison of the phosphotyrosine-containing proteins from a variety of embryonic tissues with those from Rous sarcoma virus–transformed chick embryo fibroblasts. Proteins phosphorylated on tyrosine are detectable in all the tissues with the anti–phosphotyrosine antibodies, but in substantially lower amounts than in the Rous sarcoma virus–transformed fibroblasts. Fig. 1 B shows the overall pattern of proteins from the tissues in an identical gel stained with Coomassie Blue. The specificity of the immunoblotting for phosphotyrosine in embryonic tissue proteins is demonstrated by the absence of antibody binding to embryonic thigh extracts in the presence of 10 mM phosphotyrosine, as shown in Fig. 1 C. The same concentration of phosphoserine or phosphothreonine did not affect antibody binding (Fig. 1 C). Furthermore, incubation of the thigh extracts with acid phosphatase, a treatment that removes all phosphate groups on proteins (Cooper and King, 1986), completely abolished the immunoblotting with the anti–phosphotyrosine antibodies (not shown).

We then examined the phosphorylation of proteins on tyrosine throughout the development of each of the tissues. The extent of tyrosine phosphorylation changed during development, decreasing substantially in almost all proteins just before hatching and was very low or undetectable in adult tissues. Immunoblots stained with the anti–phosphotyrosine antibodies are shown for heart, thigh, and gizzard in Fig. 2, A-C, respectively. All three tissues contain proteins at 120 and 70 kD that are heavily labeled with the anti–phos-

photyrosine antibodies and proteins at 180 and 35 kD that appear less extensively labeled. Thigh also contains a prominent tyrosine phosphorylated protein band at 60 kD, whereas in gizzard and heart this is a minor component. In both heart and thigh, the level of protein tyrosine phosphorylation decreases to a low level by day 14 of development, with the exception of the 120-kD protein band. In the gizzard, the decrease in tyrosine phosphorylation of most proteins is less pronounced between days 8 and 21.

The immunoblots of brain and lens with the anti–phosphotyrosine antibodies are shown in Fig. 3. In brain (Fig. 3 A), the substrates of tyrosine protein kinases appear to change between days 7 and 8 of development. In contrast to the other tissues examined, in brain, the major tyrosine phosphorylated proteins detected after day 8 have molecular masses >120 kD. The variation in the phosphotyrosine-containing bands in 16–20-d brain extracts was not always observed. The set of proteins phosphorylated on tyrosine in lens appears more limited than in the other tissues (Fig. 3 B). The proteins most extensively phosphorylated on tyrosine in lens have molecular masses of 120 and 60 kD and they persist throughout development, while a protein band of 210 kD contains phosphotyrosine only in 9–11-d lens.

Fig. 4, A and B shows the results of immunoblotting extracts of lung and intestine, respectively, with anti–phosphotyrosine antibodies. Lung shows a fairly uniform level of protein tyrosine phosphorylation throughout most of its development, whereas intestine contains high levels of tyrosine
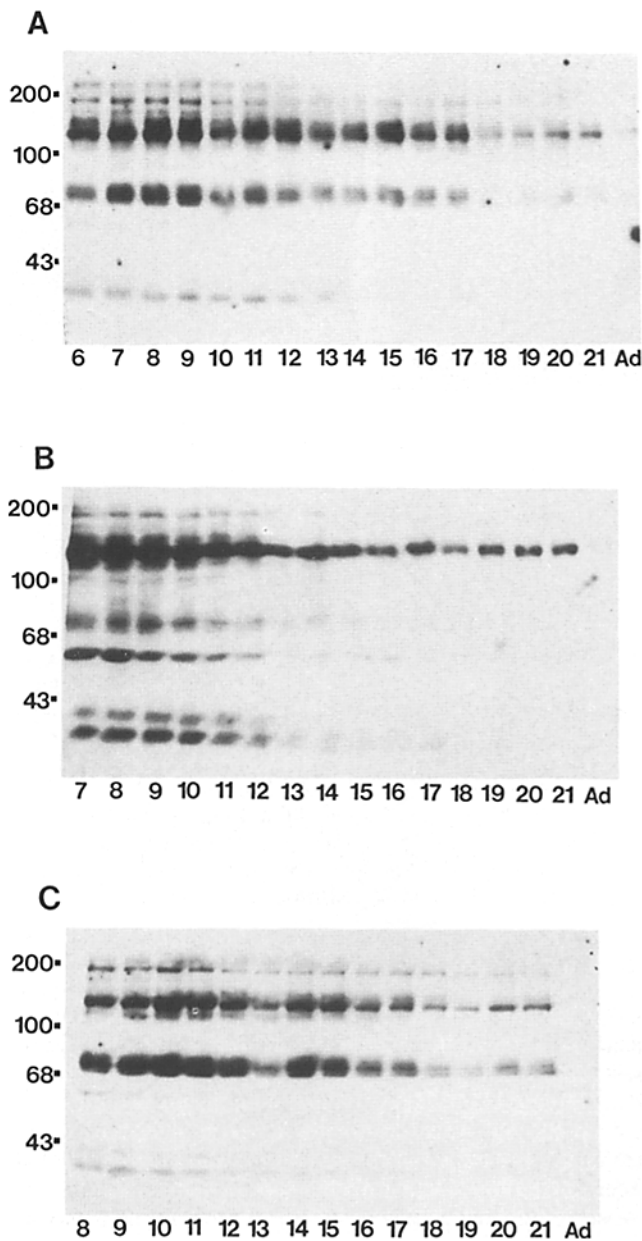
A

B

C

Figure 2. Immunoblotting of (*A*) 6–21-d embryonic and adult heart, (*B*) 7–21-d embryonic and adult thigh, and (*C*) 8–21-d embryonic and adult gizzard with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A. The days of development are noted at the bottom of each immunoblot and the molecular mass markers are indicated on the figure.

A

B

Figure 3. Immunoblotting of (*A*) 6–21-d embryonic and adult brain and (*B*) 9–21-d embryonic and adult lens with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A. The days of development are noted at the bottom of each immunoblot and the positions of the molecular mass markers are indicated by the dots at the side of the gel. Several of the molecular mass markers are also indicated on the figure.

phosphorylated proteins between days 8 and 12 and much lower levels throughout the rest of its development. In contrast to most of the other adult tissues examined, adult lung contains some proteins phosphorylated on tyrosine. Both lung and intestine contain proteins phosphorylated on tyrosine with molecular masses of 120, 70, 60, and 35 kD. The 35-kD band in lung reacts very weakly with the anti–phosphotyrosine antibody.

The immunoblots of liver and kidney with the anti–phosphotyrosine antibodies are shown in Fig. 5, *A* and *B*, respectively. The major phosphotyrosine-modified proteins in both
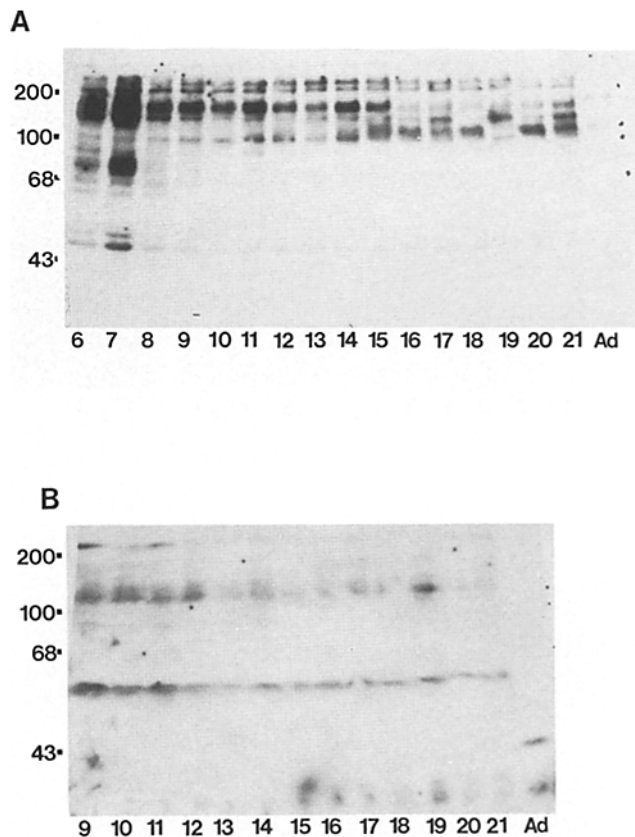
tissues have molecular masses of 120 and 70 kD. Lower levels of phosphotyrosine are seen in protein bands of 180, 150, 60, and 35 kD. The amount of protein tyrosine phosphorylation remains fairly constant throughout kidney development, but decreases after day 15 during liver development.

These results suggest that at least some of the tyrosine-phosphorylated proteins of similar molecular masses seen in the immunoblots of the different tissues may represent the same, or closely related, proteins. Data confirming this hypothesis were obtained in one-dimensional peptide mapping experiments with embryonic thigh, gizzard, intestine, heart, and lung. Bands at 120 and 70 kD were cut from an SDS-polyacrylamide gel of these tissues, applied to the top of a second polyacrylamide gel, digested with V8 protease, electrophoresed, transferred to nitrocellulose, and immunoblotted with anti–phosphotyrosine antibodies. The results are shown in Fig. 6. In the presence of 10 ng V8 protease, the 120-kD protein band of all five tissues gave closely similar patterns of three lower molecular mass phosphotyrosine-containing peptide bands (Fig. 6 *A*). Higher (15 ng) amounts of the protease resulted in complete loss of the phosphotyrosine-containing peptides, whereas lower amounts (5 ng) gave no digestion (not shown). The 70-kD band from all five tissues was digested to one major lower molecular mass
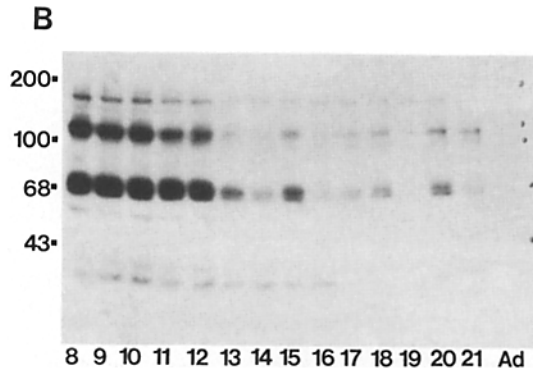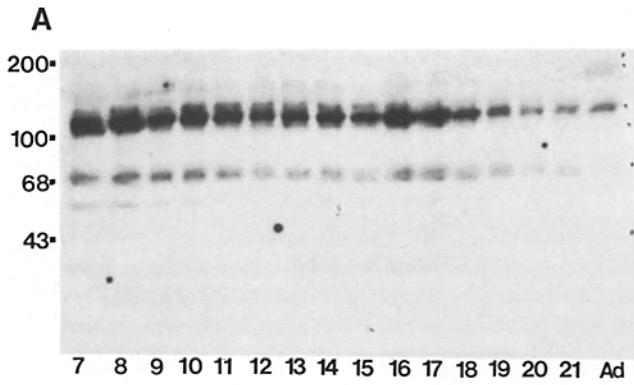
Figure 4. Immunoblotting of (*A*) 9–21-d embryonic and adult lung and (*B*) 8–21-d embryonic and adult intestine with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A. The days of development are noted at the bottom of each immunoblot and the positions of all the molecular mass markers are indicated by the dots at the side of the gel. Several of the markers are also indicated on the figure.
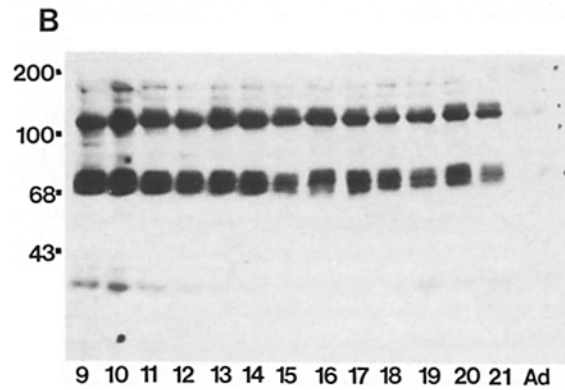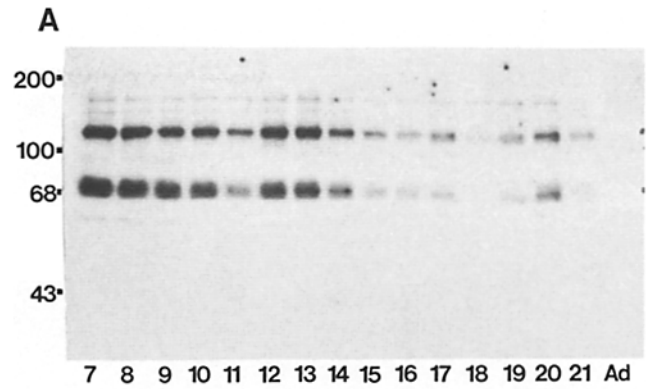

Figure 5. Immunoblotting of (*A*) 7–21-d embryonic and adult liver and (*B*) 9–21-d embryonic and adult kidney with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A. The days of development are noted at the bottom of each immunoblot and the positions of the molecular mass markers are indicated by the dots at the side of the gel. Several of the markers are also indicated on the figure.

phosphotyrosine-containing band of 68 kD with 10 ng V8 protease (Fig. 6 *B*). With 15 ng protease the same results were obtained, whereas 25 ng brought about complete loss of the phosphotyrosine-containing bands, and lower (5 ng) protease levels gave no digestion (not shown). For comparison, undigested samples of the 70-kD band are also shown (Fig. 6 *B*).

The proteins vinculin and integrin have molecular masses of ∼120 kD and contain phosphotyrosine in cells transformed by Rous sarcoma virus (Sefton et al., 1981; Hirst et
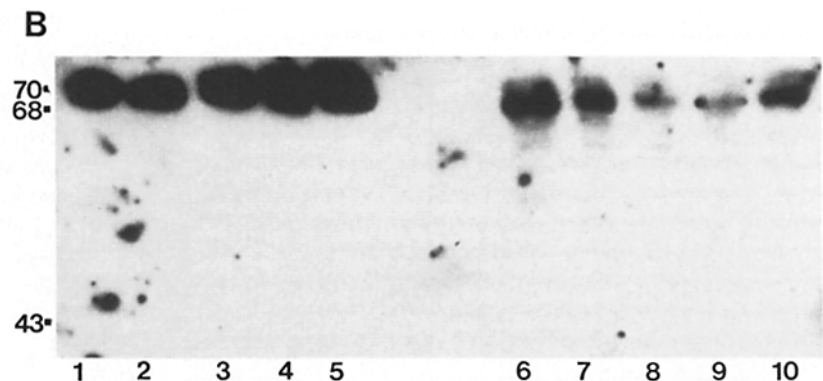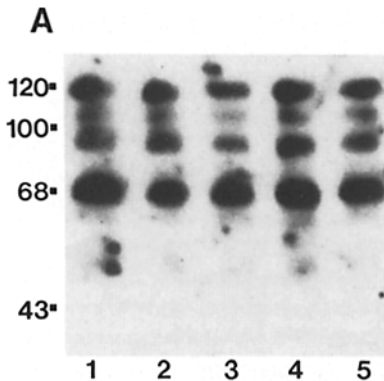

Figure 6. Immunoblotting of the V8 protease (10 ng) digested (*A*) 120 kD and (*B*) 70 kD phosphotyrosine-containing protein bands from intestine (lanes *1* and *6*), lung (lanes *2* and *7*), heart (lanes *3* and *8*), thigh (lanes *4* and *9*), and gizzard (lanes *5* and *10*) with rabbit anti–phosphotyrosine antibodies and ^{125}I-protein A.
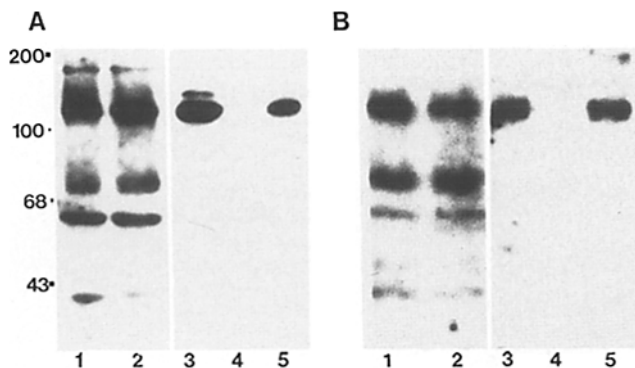
Figure 7. Immunoblotting of vinculin (*A*) and integrin (*B*) immuno-precipitation experiments with rabbit anti-phosphotyrosine anti-bodies (*A* and *B*, lanes *1* and *2*), rabbit anti-vinculin antibodies (*A*, lanes *3-5*), or mouse anti-integrin antibodies (*B*, lanes *3-5*) and ^{125}I-protein A. Extracts of 8-d embryonic chicken thigh were used for the immunoprecipitation. Lanes *1* and *3*, thigh extracts before immunoprecipitation; lanes *2* and *4*, thigh extracts completely depleted of vinculin (*A*) or integrin (*B*) by immunoprecipitation; and lane *5*, immunoprecipitated vinculin (*A*) or integrin (*B*).

al., 1986). Thus, it was of interest to determine if the major 120-kD substrate seen in the embryonic tissues was vinculin or integrin. Vinculin and integrin were immunoprecipitated from an extract of 8-d embryonic thigh and samples of the extract before and after immunoprecipitation, and the immunoprecipitated proteins were analyzed for the presence of either phosphotyrosine-containing proteins, vinculin (120 kD) or integrin (140 kD). The results are shown in Fig. 7. The removal of all of the vinculin (Fig. 7 *A*, lanes *1* and *2*) or integrin (Fig. 7 *B*, lanes *1* and *2*) did not significantly affect the reaction of the anti-phosphotyrosine antibodies with the 120-kD band. In a similar experiment, the removal of all the talin did not change the blotting pattern of the high molecular mass band with the anti-phosphotyrosine antibodies (not shown). Thus, the major phosphotyrosine-containing protein observed at 120 kD in the embryonic tissues does not appear to be either of the previously described protein tyrosine kinase substrates vinculin or integrin.

To characterize further some of the proteins phosphorylated on tyrosine in the tissues, indirect immunofluorescence labeling experiments with the anti-phosphotyrosine antibodies were carried out on primary cultures of 8-d embryonic cardiac, lens, endothelial, and gizzard cells. The cells were cultured for only 24 h after removal from the chicken embryos, before being used in the immunofluorescence experiments. The results are shown in Fig. 8. All four cell types show strong immunofluorescence labeling with the anti-phosphotyrosine antibodies, at sites of microfilament-membrane interaction, suggesting that at least one of the proteins seen in the immunoblots is localized to these sites. The immunofluorescence labeling results also indicate that proteins phosphorylated on tyrosine are present in cardiac muscle cells, in lens epithelial cells, in gizzard endothelial cells, and in gizzard smooth muscle cells, after such cells have been removed from their respective tissues and cultured for a short time period. The immunofluorescence labeling of the cardiac muscle cells with the anti-phosphotyrosine antibodies was examined as a function of time in culture (not

shown). Strong immunofluorescence labeling of cell-cell and cell-substrate contact sites was observed at the earliest times (12 h) tested. The intensity of the immunofluorescence labeling with the anti-phosphotyrosine antibodies decreased after 3 d in culture.

Discussion

The availability of high affinity antibodies to phosphotyrosine has made it possible for the first time to survey developing embryonic tissues in vivo for the presence and characteristics of proteins phosphorylated on tyrosine. Since the alternative approach to the analysis of phosphotyrosine-containing proteins in tissues requires the labeling of cells with ^{32}P-orthophosphate in vivo, followed by differential hydrolysis of the phosphoamino acids and autoradiography, this type of study has not been previously feasible. A number of anti-phosphotyrosine antibodies have been generated in different ways (Ross et al., 1981; Frackelton et al., 1983; Comoglio et al., 1984; Ek and Heldin, 1984; Pang et al., 1985; Wang, 1985), but that produced by Wang (1985), used in this study and in the following paper (Takata and Singer, 1988), has proven particularly useful. This anti-phosphotyrosine antibody is specific for phosphotyrosyl residues in proteins when used for immunoprecipitation (Wang, 1985), immunoblotting (Wang, 1985; Pasquale et al., 1986; Morla and Wang, 1986), and immunofluorescence microscopy (Maher et al., 1985). In addition, it recognizes a number of known tyrosine phosphorylated proteins including vinculin, talin, and pp60^{v-src} in Rous sarcoma virus-transformed cells (Pasquale et al., 1986; unpublished results), and the PDGF receptor in PDGF-stimulated BALB/c 3T3 cells (Wang, 1985).

The immunoblotting results show that tyrosine phosphorylated proteins are present in all the embryonic chicken tissues examined from 7-21 d of development in ovo, but are generally reduced or absent in the same tissues taken from adult chickens. The decrease in the extent of protein tyrosine phosphorylation during development is not an artifact due to increased dephosphorylation of phosphotyrosyl residues occurring as the protein concentration at the time of tissue homogenization increases. As the size of the tissues increased with the age of the embryos, larger volumes were used for homogenization, so that the tissue protein concentrations and, therefore, the concentration of phosphotyrosine phosphatases remained constant. In addition, in control experiments (not shown), the amount of phosphotyrosine in protein samples from 10-d thigh homogenized over a 10-fold range of protein concentrations was examined and found to be constant.

There are a limited number of major tyrosine phosphorylated protein bands identified by the anti-phosphotyrosine antibodies in all the tissues examined, ranging in molecular mass from 35 to 220 kD. However, there may be other proteins phosphorylated on tyrosine residues which we do not detect, either because the antibodies do not recognize them or because they are present at too low a concentration.

The embryonic substrates of tyrosine kinase activity that we detect are proteins that are currently unidentified. The major high molecular mass tyrosine phosphorylated proteins in embryonic tissues are not the previously reported pp60^{v-src} substrates, vinculin (120 kD; Sefton et al., 1981), talin (215
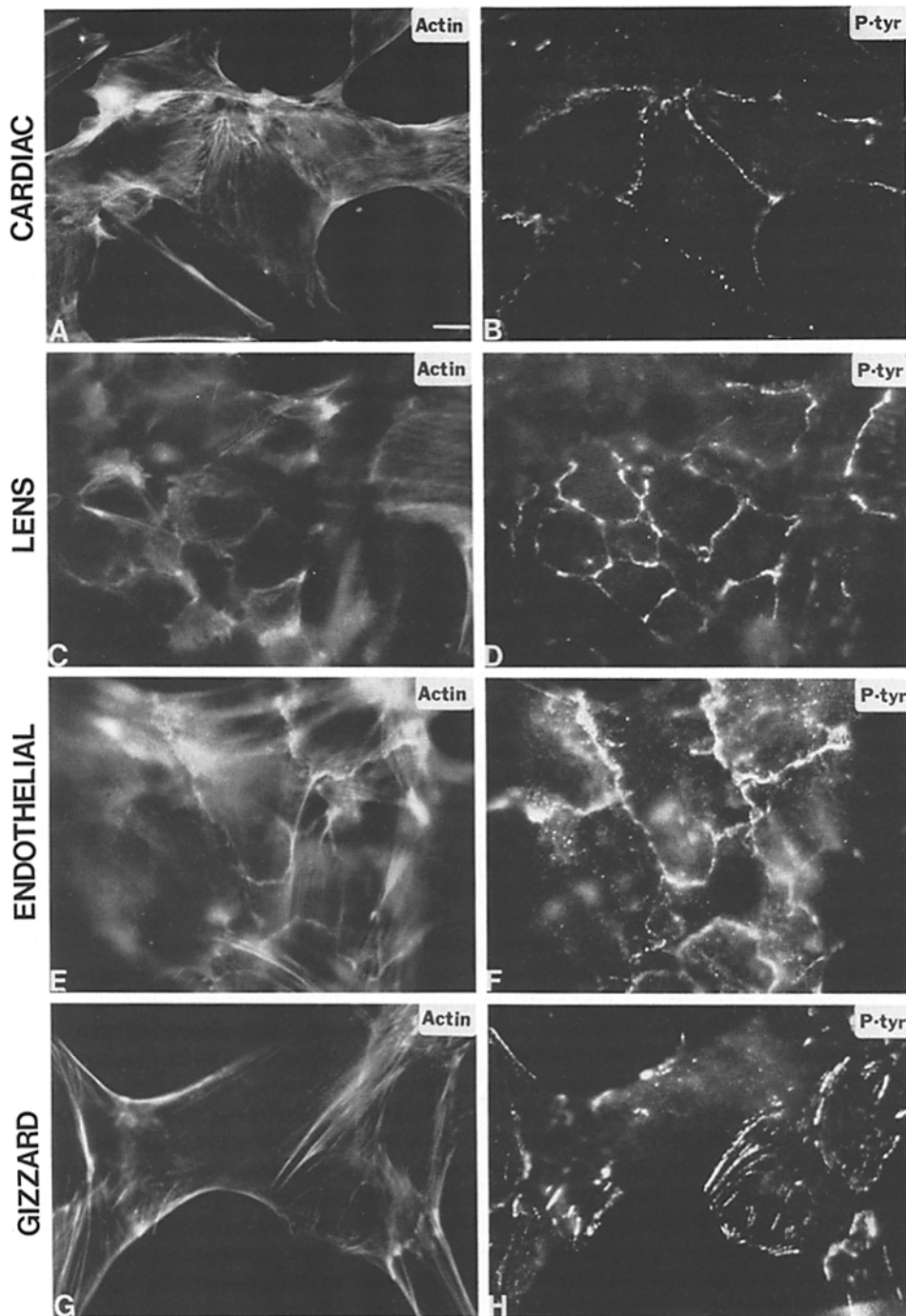
Figure 8. Indirect immunofluorescence labeling of primary cultures of embryonic cardiac muscle (*A, B*); lens (*C, D*); endothelial (*E, F*); and gizzard (*G, H*) cells with rabbit anti–phosphotyrosine antibodies followed by rhodamine-conjugated goat anti–rabbit IgG (*A, C, E, G*) and NBD-phallacidin to label F-actin (*B, D, F, H*). Bar, 1 μm.

kD; Pasquale et al., 1986), or integrin (140 kD; Hirst et al., 1986). Immunoprecipitation of all the vinculin, talin, or integrin from an extract of embryonic thigh did not reduce the level of protein tyrosine phosphorylation of the remaining material. In addition, phosphotyrosine was not detected in the immunoprecipitated proteins (not shown), indicating that <1% of each of these proteins could be tyrosine phosphorylated in the embryonic thigh extract. This is based on the observation that the phosphotyrosine in the same amount of vinculin immunoprecipitated from Rous sarcoma virus-transformed cells is barely detectable with the anti–phosphotyrosine antibodies and 1% of the vinculin molecules contain

phosphotyrosine in these transformed cells (Sefton et al., 1981).

The finding that proteins of similar molecular mass are tyrosine phosphorylated in many different tissues during development suggests that these may be related or identical proteins. When the immunoblots for phosphotyrosine-containing proteins from the different tissues are compared, bands with apparent molecular masses of 120 and 70 kD are tyrosine phosphorylated in almost all cases (Fig. 1 *A*). Our studies (Fig. 6) with the 120-kD tyrosine phosphorylated protein bands indicate a close structural relationship among the phosphotyrosine-containing proteins of 120 kD from the

different tissues since they are proteolyzed into similar phosphotyrosine-containing peptides. Similar results were obtained for the 70-kD phosphotyrosine-containing protein bands from the different tissues. It was recently proposed (Hunter, 1987) that a large number of protein tyrosine kinases have yet to be discovered. One or more of the tyrosine phosphorylated proteins seen in the embryonic tissues may be an unidentified autophosphorylating protein tyrosine kinase. It is also possible that some of the proteins phosphorylated on tyrosine in different tissues are growth factor receptors with intrinsic tyrosine kinase activity such as the receptors for PDGF, epidermal growth factor, insulin, or other unidentified growth factors. Interaction of the growth factor with the receptor would activate the protein tyrosine kinase stimulating autophosphorylation of the receptor. The protein tyrosine kinase activity associated with several different growth factor receptors has been detected during embryonic development (Hortsch et al., 1983; Peyron et al., 1985; Adamson, 1987).

The overall level of protein tyrosine phosphorylation in the tissues may reflect the sum of the activities of both protein tyrosine kinases and phosphotyrosine phosphatases. Several groups have identified protein tyrosine kinases in adult tissues, including pig brain (Braun et al., 1986), rat liver (Wong and Goldberg, 1983), and rat spleen (Swarup et al., 1983). However, there may be very active phosphotyrosine phosphatases in adult tissues such that the turnover of phosphate on tyrosyl residues is extremely rapid.

Our results show that the overall level of protein tyrosine phosphorylation in the tissues changes during development. There appear to be three general patterns of variation. In some tissues, such as brain, thigh, and intestine, a peak of protein tyrosine phosphorylation is observed during the earlier part of development. In other tissues, such as heart, gizzard, and liver, there is a gradual decrease in the amount of phosphotyrosine in proteins during development. In kidney, lung, and lens, fairly uniform levels of protein tyrosine phosphorylation are found throughout development. While many of the phosphotyrosine-containing proteins in a single tissue show similar kinetics in the changes of their levels of phosphotyrosine during development, not all proteins do. It is not clear whether the amounts of the phosphotyrosine-containing proteins or the level of phosphotyrosine in the proteins are changing. The resolution of this question will require antibodies to the proteins.

Our immunofluorescence labeling results with the anti-phosphotyrosine antibodies and primary cultures of embryonic cardiac, lens, gizzard smooth muscle, and endothelial cells are consistent with the immunoblotting data presented, which show that proteins phosphorylated on tyrosine are present in extracts of embryonic heart, lens, and gizzard. The concentration of the proteins phosphorylated on tyrosine at the sites of cell–cell and cell–substrate interaction in primary cultures of embryonic cells is in good agreement with the results obtained by Takata and Singer (1988) using frozen sections of embryonic tissues as described in the following paper. Using both immunofluorescence and immunoelectron microscopy, they detected high concentrations of proteins containing phosphotyrosine in close proximity to the cell membrane in endothelial, lung, and lens cells. However, they did not detect the presence of proteins phosphorylated on tyrosine in frozen sections of embryonic cardiac muscle

cells. This difference between our data and the data of Takata and Singer (1988) may be explained by differences in the accessibility of the phosphotyrosine-containing proteins in cultured cells and tissue sections to the anti–phosphotyrosine antibodies. In addition, the immunofluorescence labeling experiments may visualize only a fraction of the phosphotyrosine-containing proteins detected in the tissues by immunoblotting. For example, proteins phosphorylated on tyrosine, which are distributed diffusely throughout the cytoplasm, would probably not appear prominent by immunofluorescence microscopy.

What is the significance of the changes in the overall levels of protein tyrosine phosphorylation in the different tissues that are seen during development? It is known from studies on growth factors that protein tyrosine phosphorylation increases following stimulation of quiescent cells to divide Pasquale, E. B., P. A. Maher, and S. J. Singer, manuscript submitted), suggesting that tyrosine phosphorylated proteins may be important for cell division during development. This is consistent with the very low levels of phosphotyrosine-containing proteins present in most of the adult tissues examined. However, the high levels of phosphotyrosine-containing proteins in the embryonic tissues do not correlate simply with times of active cell proliferation. The changes in the amounts of the major phosphotyrosine-modified proteins may also play a role in the control of cellular differentiation, as was suggested by the studies on pp60$^{c\text{-}src}$ in the nervous system (Sorge et al., 1984; Fults et al., 1985).

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