Paxillin Is a Major Phosphotyrosine-containing Protein during Embryonic Development

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Abstract. Phosphotyrosine-containing proteins were immunoprecipitated from embryonic chicken tissue extracts using anti-phosphotyrosine antibody coupled to agarose beads. Major phosphotyrosine-containing proteins of 110, 70, and 50 kD were observed following blotting with anti-phosphotyrosine antibody. The 70-kD band was selectively removed from the samples by precipitation with antibodies to the focal adhesion protein paxillin, therefore identifying paxillin as one of the major tyrosine kinase substrates during chick embryonic organogenesis. The tyrosine phosphorylation of paxillin is regulated developmentally: during embryogenesis, a marked decrease in its phosphotyrosine content was observed, although the total level of paxillin remained essentially constant. Approximately 20% of the paxillin was phosphorylated on tyrosine in the early embryo. In contrast, tyrosine phosphorylation of paxillin was undetectable in the adult. A similar profile of phosphotyrosine-containing proteins was identified in rat embryos. Paxillin was also found to be a major phosphotyrosine-containing protein in the rat embryo. These data suggest that the regulated phosphorylation of tyrosine residues on paxillin may perform a critical role in controlling cell and tissue cytoarchitecture rearrangement during vertebrate development.

PRECISE control of cell division, differentiation, and growth is essential for normal embryonic tissue development. Evidence that tyrosine kinases may play a pivotal role in coordinating these events has come from the recognition that the rapid, uncontrolled cell division of certain virally transformed cells grown in culture is a direct result of the over expression of the viral oncogenes that encode proteins with tyrosine kinase activity (Hunter and Sefton, 1980; Gentry and Rohrschneider, 1984; Bishop, 1985; Hunter and Cooper, 1985). As a result, many developmental studies have concentrated on the identification of normal cellular tyrosine kinase homologues of the viral oncogenes (reviewed in Adamson, 1987). These proto-oncogenes that encode tyrosine kinases fall into two classes. Firstly, there are receptor tyrosine kinases (Yarden and Ullrich, 1988) which include, for example, EGF receptor/c-erbB (Adamson, 1983; Ushiro and Cohen, 1985), and colony stimulating factor-1 (CSF-1) receptor/c-fms (Muller et al., 1983; Rettenmier et al., 1985; Coussens et al., 1986). Secondly, there are nonreceptor tyrosine kinases (e.g., c-src, c-abl) (Takeya and Hanafusa, 1983; Wyke, 1983; Sejerson et al., 1986). Of the latter class, $pp60^{c\text{-}src}$, the normal cellular homologue of the Rous sarcoma virus (RSV)[1] gene product $pp60^{v\text{-}src}$ has been studied in the greatest detail. It is expressed at highest levels in tissues of neuronal origin (Cotton and Brugge, 1983; Levy et al., 1984; Sorge et al., 1984). It is particularly concentrated in the nerve growth cones (Maness et al., 1988) where tubulin has been shown to be one of its substrates (Matten et al., 1990).

The advent of anti-phosphotyrosine antibodies has enabled the visualization of tyrosine kinase substrates during tissue development (Maher and Pasquale, 1988). In such studies a temporal appearance of phosphotyrosine-containing proteins was observed, with high levels of phosphotyrosine being recorded during the early stages of development and protein phosphotyrosine levels tapering off to zero or close to zero in the adult tissues (Maher and Pasquale, 1988). However, the identity and significance of these phosphotyrosine-containing proteins that undergo such dramatic changes in phosphotyrosine content during development has remained undetermined.

Paxillin is a 68-kD cytoskeletal protein that localizes to the focal adhesions of cultured cells (Turner et al., 1990). It is potentially involved in actin-membrane attachment at these sites via an interaction with vinculin (Turner et al., 1990). Paxillin has previously been shown to be heavily phosphorylated on tyrosine residues in chick embryo fibroblasts transformed by RSV (Glenney and Zokas, 1989). During transformation, major reorganization of the actin-based cytoskeleton is observed in association with the disassembly of cell-extracellular matrix linkages (Burridge, 1986). Since reorganization of the cytoskeleton is a prerequisite to cell division, growth, and differentiation during embryogenesis this present study has focused on determining if tyrosine

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1. *Abbreviation used in this paper*: RSV, Rous sarcoma virus.

phosphorylation of paxillin may also occur during tissue development.

Materials and Methods

Tissue Extract Preparation

Tissues were removed from chicken embryos of the appropriate age and rinsed briefly in HBSS. The tissue was homogenized on ice in a Dounce homogenizer in extraction buffer (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% deoxycholate, 50 μM Na Vanadate, 10 μM leupeptin). The homogenate was clarified by centrifugation at 100,000 *g* for 30 min. Protein extracts of the torsos of day 14 rat embryos were prepared as described for the chick embryo tissue extracts. The protein concentration of dilutions of the extracts was determined by reading the absorbance at 280 nm. When a direct comparison of either paxillin levels or protein-phosphotyrosine levels was to be performed between extracts of different developmental stages gel samples were prepared accordingly to ensure equal amounts of protein for each of the stages.

Immunoprecipitations

The clarified tissue extracts were incubated and mixed end over end with anti-phosphotyrosine antibody (PY20) (Glenney et al., 1988) coupled to agarose beads for 90 min at 4°C. The beads were sedimented at 10,000 *g* and washed extensively in extraction buffer. Phosphotyrosine-containing proteins were released from the beads by incubation for 30 min on ice in extraction buffer containing 10 mM phenyl phosphate and 0.1 mg/ml ovalbumin as carrier protein. The beads were pelleted and the supernatant removed either for gel analysis or for immunoprecipitation with anti-paxillin antibodies. For the latter, the supernatant was incubated with end over end mixing at 4°C with mAb 165 (Glenney and Zokas, 1989; Turner et al., 1990) to paxillin coupled to agarose beads for 90 min. The immunobeads were subsequently washed thoroughly in extraction buffer before gel analysis. In certain experiments paxillin was precipitated directly from the tissue extracts using anti-paxillin antibody coupled to agarose beads.

SDS-PAGE and Western Blot Analysis

Protein samples of tissue extracts or immunoprecipitates were electrophoresed on 10% gels according to Laemmli (1970) with a bis acrylamide concentration of 0.13% and either stained with Coomassie blue or transferred to nitrocellulose as previously described (Towbin et al., 1979). Phosphotyrosine-containing protein bands were visualized, following blocking of the nitrocellulose, by incubation with ^{125}I-labeled anti-phosphotyrosine antibody (PY20) (7×10^5 cpm/ml) for 60 min. Filters were washed and processed for autoradiography. Paxillin was identified by incubation of the filter with a primary layer of mAb to paxillin followed by ^{125}I-labeled rabbit anti-mouse immunoglobulin (7×10^5 cpm/ml). Antibody iodinations were performed using Iodogen (Pierce Chemical Co., Rockford, IL) (Fraker and Speck, 1978).

Semi-quantitative Analysis of Paxillin Tyrosine Phosphorylation

Extracts of gizzard tissue taken at different stages in development were prepared as described above. The samples were normalized for protein content by measuring the OD_{280} as above. Duplicate samples for each stage were either incubated with an excess of anti-phosphotyrosine antibody-agarose beads to precipitate the phosphotyrosine-containing paxillin (in addition to the other phosphotyrosine-containing proteins) or with an excess of anti-paxillin-agarose beads to precipitate all of the paxillin in the sample. The samples were electrophoresed, transferred to nitrocellulose, and blotted with antibody to paxillin followed by radio-iodinated anti-mouse antibody. The amount of paxillin in each of the samples was then determined indirectly by excision of the radioactive bands and counting in a gamma counter. Background counts determined from an equal sized piece of nitrocellulose were subtracted from each value. The percentage of paxillin containing phosphotyrosine was determined by the ratio of the number of counts in the two samples.

Results

Phosphotyrosine-containing proteins were immunoprecipitated from day 8 chicken embryonic gizzard smooth muscle extracts using anti-phosphotyrosine antibody (PY20) (Glenney et al., 1988) coupled to agarose beads. The bound phosphotyrosine-containing proteins were subsequently eluted specifically from the antibody by incubation of the immune complex in 10 mM phenylphosphate. Western blot analysis of the eluted proteins with radioiodinated anti-phosphotyrosine antibody revealed three major phosphotyrosine-containing protein bands with molecular weights of ~110, 70, and 50 kD (Fig. 1 *a*, lane *1*), in addition to a number of more minor species. To determine if the phosphoprotein of 70 kD was paxillin (previously shown to be most abundant in smooth muscle tissue; Turner et al., 1990), the mixture of phosphotyrosine-containing proteins was incubated with anti-paxillin antibody coupled to agarose beads. Analysis by anti-phosphotyrosine blotting of the resulting supernatant and pellet from the immunoprecipitation revealed that the complement of phosphotyrosine-containing proteins had been selectively depleted of the majority of the diffuse 70-kD band (Fig. 1 *a*, lane *3*). The 70-kD band was enriched accordingly in the pellet (Fig. 1 *a*, lane *2*). The presence of paxillin in the extract eluted from the anti-phosphotyrosine beads was confirmed by Western blotting with antibody to paxillin (Fig. 1 *b*).

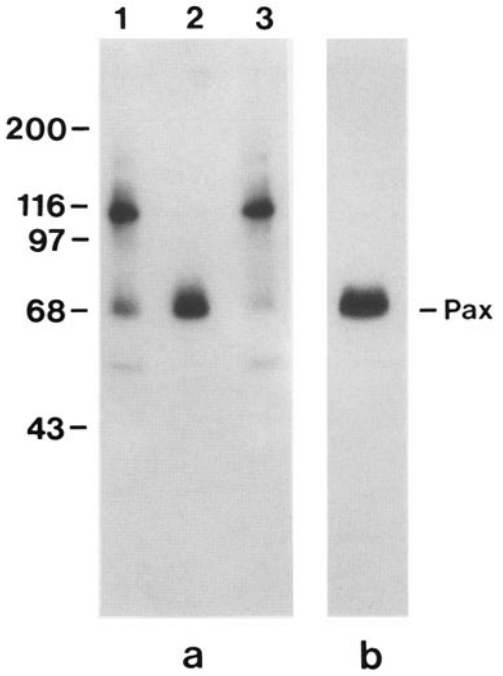

Figure 1. Paxillin is one of the major phosphotyrosine-containing proteins in the early chick embryo gizzard. (*a*) Anti-phosphotyrosine blot of phosphotyrosine-containing proteins precipitated from a day 8 chick embryo gizzard extract (lane *1*). Three major bands could be identified with molecular weights of ~110, 70, and 50 kD. The 70 kD species was identified as paxillin by selective removal of this band from the phosphotyrosine protein mixture by immunoprecipitation with anti-paxillin antibody (lane *2*). (lane *3*) Paxillin-depleted phosphotyrosine protein sample. (*b*) Anti-paxillin blot of phosphotyrosine-containing proteins precipitated from the gizzard extract confirming the presence of paxillin. Relative molecular mass standards ($M_r \times 10^{-3}$) are shown to the left.

To analyze the levels of protein tyrosine phosphorylation at different embryonic stages, extracts containing equal

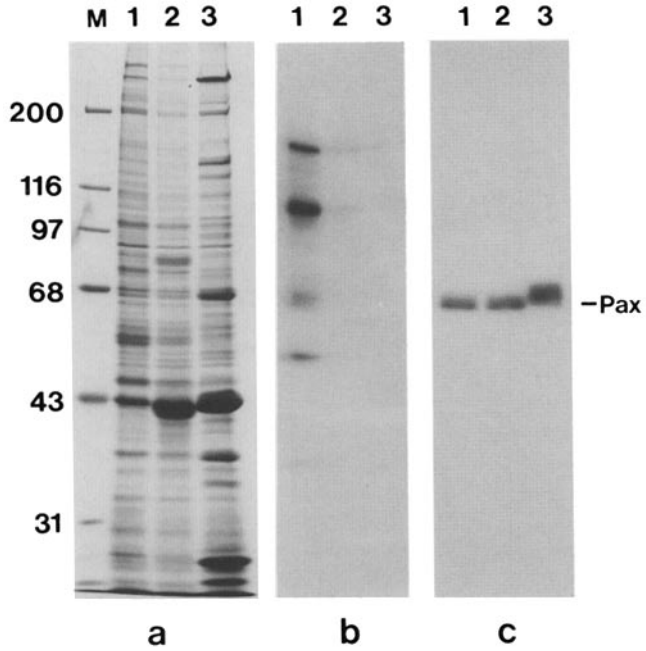

Figure 2. Comparison of phosphotyrosine and paxillin levels during chick embryo gizzard development. Equal protein loadings of gizzard extract from day 8 (lanes *1*) and day 20 (lanes 2) embryos and adult tissue (lanes *3*) were co-electrophoresed on 10% gels and either stained with Coomassie blue (*a*) or transferred to nitrocellulose and blotted with either anti-phosphotyrosine antibody (*b*) or anti-paxillin antibody (*c*). While there was an elevated level of phosphotyrosine-containing proteins in the day 8 embryo (*b*, lane *1*), the level of phosphotyrosine was almost zero just before hatching (*b*, lane *2*) and was absent from the adult (*b*, lane *e*) at this level of detection. In contrast, the level of paxillin remained essentially constant prior to hatching and demonstrated slightly elevated levels in the adult (*c*). The major protein band at 68 kD stained with Coomassie blue in the adult tissue (*a*, lane *3*) is not related to paxillin. *M*, in *a*, molecular weight standards.

amounts of protein from embryonic day 8, day 20, and adult gizzard were blotted with anti-phosphotyrosine antibody. This revealed that the elevated level of phosphotyrosine observed on proteins in embryonic day 8 tissue diminished considerably by day 20 and was essentially absent in the sample of adult tissue (Fig. 2 *b*) as described previously (Maher and Pasquale, 1988). An additional major phosphotyrosine-containing band of approximately 170 kD was observed in the day 8 tissue sample which was poorly immunoprecipitated with the anti-phosphotyrosine antibody beads (c.f. Fig. 1 *a*, lane *1* and Fig. 2 *b*, lane *1*).

The change in protein phosphotyrosine levels during embryogenesis could be due either to a decrease in the amount of the respective phosphoproteins or to altered levels of phosphorylation/dephosphorylation. The relative contribution of each of these events to the overall level of tyrosine phosphorylation at different stages in development varies from tissue to tissue (Maher, 1991) and is likely also to be substrate dependent. Screening the same samples with anti-paxillin antibody demonstrated that the total level of paxillin remains essentially constant prior to hatching (Fig. 2 *c*, lanes *1* and *2*) indicating that the decrease in phosphotyrosine, at least for paxillin, is due to either decreased kinase or increased phosphatase activity. The level of paxillin was slightly elevated in the adult gizzard sample over the embryonic samples and

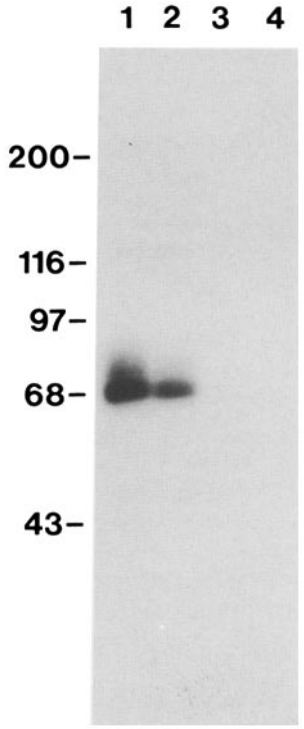

Figure 3. The tyrosine phosphorylation of paxillin decreases during development. All the paxillin was precipitated with anti-paxillin antibody from equal amounts of extracts from embryonic day 8, 16, 20, and adult gizzard. The paxillin that contained phosphotyrosine in these precipitates was visualized by blotting with anti-phosphotyrosine antibody. Lane *1*, day 8. Lane *2*, day 16. Lane *3*, day 20. Lane *4*, adult. The level of tyrosine phosphorylated paxillin decreased from day 8 through day 16 and was absent at the later stages analyzed.

the adult paxillin also demonstrated a greater heterogeneity in molecular weight (Fig. 2 *c*, lane *3*). The reason for this heterogeneity is unclear, although numerous isoforms of paxillin have been identified in adult gizzard tissue which

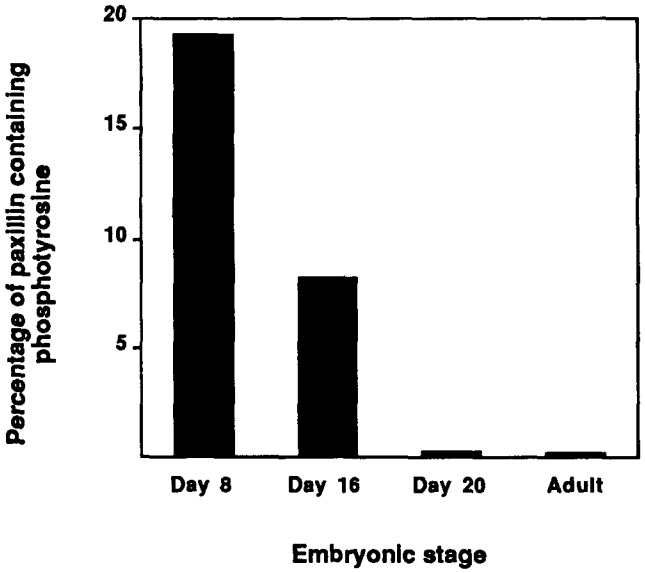

Figure 4. Quantitation of the content of phosphotyrosine in paxillin at various stages in development. Each bar represents the mean of at least three experiments. The level of paxillin phosphotyrosine was measured indirectly by precipitating paxillin from equal amounts of gizzard tissue extracts (see Materials and Methods) with either anti-phosphotyrosine antibody or anti-paxillin antibody and then blotting both samples with anti-paxillin antibody followed by ^{125}I-anti-mouse antibody. Radioactive bands, visualized by exposure to x-ray film, were excised and counted and the percentage of paxillin containing phosphotyrosine determined by the ratio between the two readings.

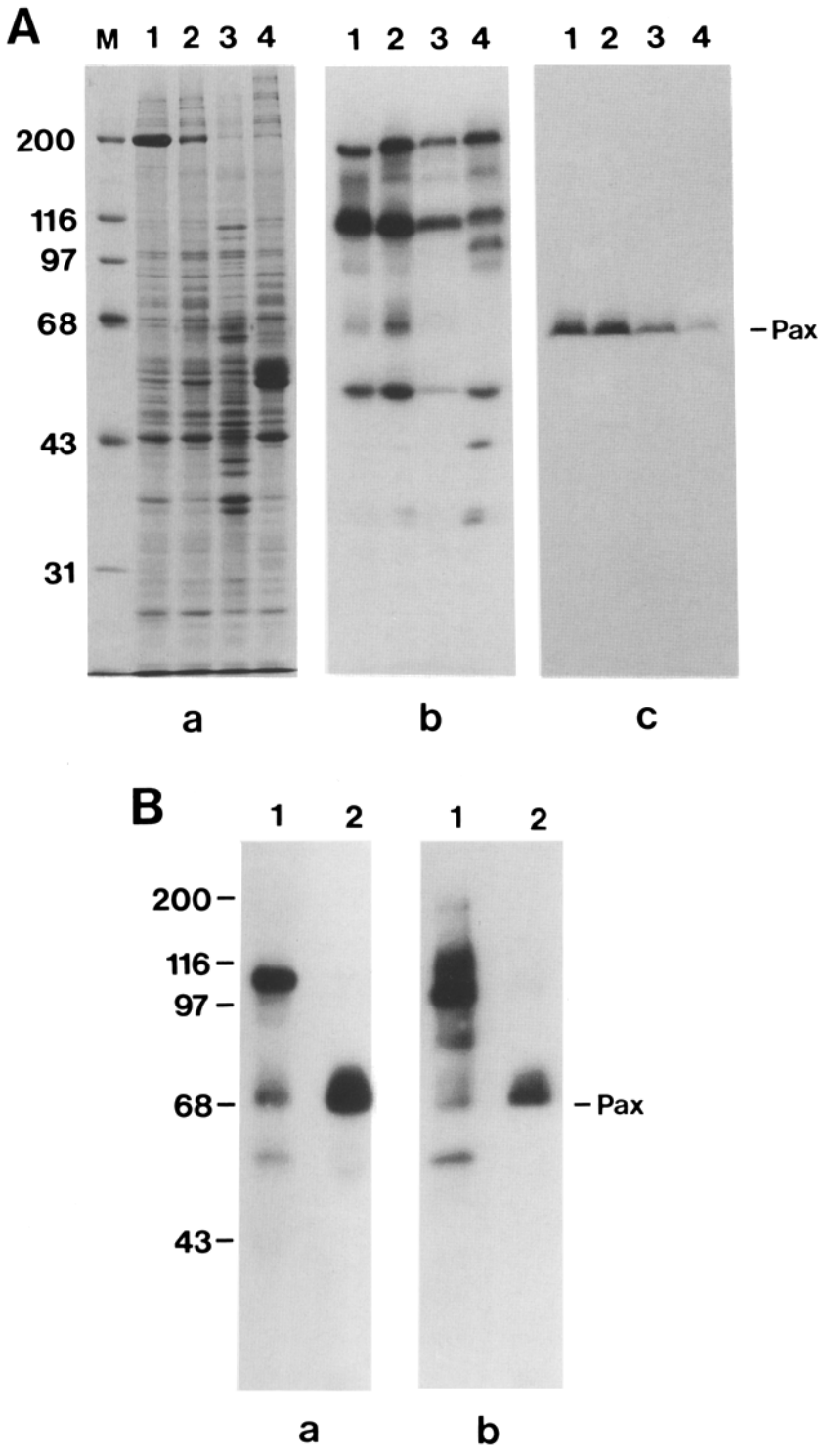

Figure 5. The phosphorylation of paxillin on tyrosine residues in the early chicken embryo is not restricted to gizzard smooth muscle. (*A*) Equal protein loadings of extracts of cardiac (lanes *1*) and skeletal muscle (lanes *2*), liver (lanes *3*) and brain (lanes *4*) taken from a day 8 embryo were electrophoresed and either stained with Coomassie blue (panel *a*), probed with anti-phosphotyrosine antibody (panel *b*) or with anti-paxillin antibody (panel *c*). Similar protein phosphotyrosine profiles were observed between tissues. However, the higher level of the 70-kD band in the cardiac and skeletal muscle samples (panel *b*, lanes *1* and *2*) reflects the higher abundance of paxillin in these two tissues (panel *c*, lanes *1* and *2*) compared with the liver or brain (lanes *3* and *4*). (*B*) Anti-phosphotyrosine blot of proteins immunoprecipitated from tissue extracts of cardiac muscle (panel *a*) and brain (panel *b*) with anti-phosphotyrosine antibody (lanes *1*) and then subsequently with anti-paxillin antibody (lanes *2*) confirmed the presence of phosphotyrosine on paxillin isolated from these two tissues. (A quantitative comparison should not be drawn from the data in *B*). Comparable results were obtained from skeletal muscle and liver (not shown). *M*, molecular weight standards.

may be because of additional posttranslational modifications (Turner et al., 1990).

To confirm that the decrease in the 70-kD anti-phosphotyrosine immunoreactive band observed in Fig. 2 *b* was due to a decrease in the level of paxillin tyrosine phosphorylation all the paxillin was precipitated directly from equal quantities of extracts of embryonic day 8, 16, 20, and adult gizzard using anti-paxillin antibody coupled to agarose beads. The tyrosine-phosphorylated paxillin contained within these precipitates was visualized by Western blotting with anti-phosphotyrosine antibody (Fig. 3). As predicted, a decrease in the amount of tyrosine phosphorylated paxillin was observed during gizzard embryogenesis.

A semi-quantitative analysis was performed to determine approximately how much paxillin was phosphorylated on tyrosine in the day 8 embryonic gizzard samples compared to later stages in development (see Materials and Methods for details). Approximately 20% of the precipitable paxillin

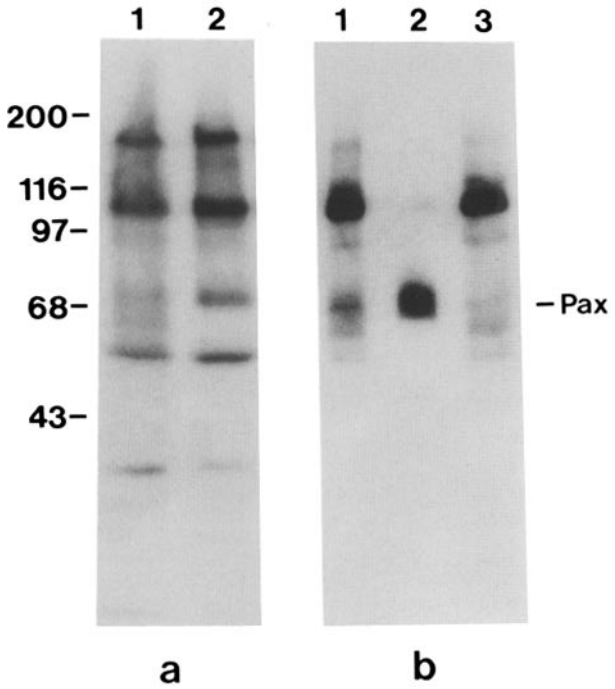

Figure 6. Paxillin is also phosphorylated on tyrosine in the rat embryo. (*a*) Anti-phosphotyrosine blot of 14 day rat embryo (torso) extract (lane *1*), and embryonic day 8 chicken gizzard extract (lane *2*). Note the strong similarity in the profiles of the two samples. (*b*) Anti-phosphotyrosine blot of: lane *1*, proteins immunoprecipitated from the day 14 rat embryo extract using anti-phosphotyrosine antibody; lane *2*, protein immunoprecipitated from the phosphotyrosine protein-enriched sample using anti-paxillin antibody; and lane *3*, paxillin-depleted phosphotyrosine protein sample.

from the total gizzard extract of the day 8 embryo was phosphorylated on tyrosine, compared to ~8% at day 16. In agreement with the blotting analysis, tyrosine phosphorylation of paxillin was undetectable in embryonic day 20 and in adult tissue (Fig. 4).

To determine whether the tyrosine phosphorylation of paxillin was restricted to gizzard tissue or if it also occurred in other tissues, extracts of skeletal muscle, cardiac muscle, liver, and brain were electrophoresed and blotted with anti-phosphotyrosine antibodies. In day 8 embryos, a pattern of phosphotyrosine-containing proteins comparable to that observed in embryonic gizzard of the same age was detected in all of these tissues (Fig. 5 *A*, panel *b*). The 70-kD phosphotyrosine-containing band was more prominent in the extracts of muscle tissue (Fig. 5 *A*, panel *b*, lanes *1* and *2*), consistent with the relative abundance of paxillin in these tissues compared to liver and brain (Fig. 5 *A*, panel *c*). The identity of the 70-kD band as paxillin was confirmed in each case by immunoprecipitation of the phosphotyrosine-containing proteins followed by anti-paxillin immunoprecipitation (c.f. cardiac muscle and brain; Fig. 5 *B*).

Paxillin phosphorylation was readily detected in extracts of whole day 5 chicken embryo torsos when assayed by the same procedures (data not shown), indicating that the phosphorylation of paxillin on tyrosine occurs early in development. The other three peptides of 170, 110, and 50 kD were also tyrosine phosphorylated in this sample. The level of tyrosine phosphorylation on paxillin in earlier embryos has not been determined due to technical limitations.

In an attempt to assess whether the profile of phosphotyrosine-containing proteins, with particular reference to paxillin, was reproduced in other organisms a comparison between extracts of chicken embryo gizzard (day 8) with rat embryo torso (day 14) was performed. Anti-phosphotyrosine blotting of these two extracts revealed a strikingly similar complement of phosphotyrosine-containing proteins with major bands of 170, 110, 70, and 50 kD (c.f. Fig. 6, panel *a*, lanes *1* and *2*). Furthermore, most of the phosphotyrosine-containing 70-kD protein band in these rat embryos was selectively immunoprecipitated with anti-paxillin antibody from this sample (Fig. 6, panel *b*, lane *2*) indicating that paxillin is also a major phosphotyrosine-containing protein in mammalian embryos. Although the small size of the rat embryos compared to those of the chicken prevents the study of the tyrosine phosphorylation of paxillin in individual rat embryo tissues the profiles obtained from the whole torso indicates that a direct comparison can be made with individual chicken tissues. Similar to the chicken, the level of phosphotyrosine was found to be very low in a number of adult rat tissues including heart, thigh, and liver (data not shown).

Discussion

In contrast to the complex pattern of total cell protein, a strikingly simple pattern of phosphotyrosine-containing proteins was observed in a variety of embryonic chicken tissues. These profiles are similar in complexity to a previous study which used a different anti-phosphotyrosine antibody for detection (Maher and Pasquale, 1988), suggesting that the profiles obtained are an accurate reflection of the major phosphotyrosine-containing proteins in these tissues. Undoubtedly there are other proteins that are phosphorylated on tyrosine that are either less abundant or less heavily phosphorylated that were not detected. For example, locally elevated concentrations of phosphotyrosine-containing proteins such as the acetylcholine receptor at the adult neuromuscular junction (Huganir and Greengard, 1990) are likely to go undetected.

The potential importance of the phosphotyrosine-containing proteins in chicken embryo development is reinforced by the detection of an almost identical complement of phosphotyrosine-containing proteins in the whole rat embryo torso. In this paper the major phosphotyrosine-containing protein of approximately 70 kD in both the chicken and the rat embryo has been shown to be the cytoskeletal protein paxillin. The identity of the remaining phosphotyrosine-containing proteins, other than paxillin, is unknown. They could be either tyrosine kinases, which undergo autophosphorylation (Hunter and Cooper, 1985), or tyrosine kinase substrates, like paxillin (which is not itself a tyrosine kinase; C. Turner, unpublished results). The other focal adhesion proteins, talin, vinculin, and integrin are not heavily phosphorylated on tyrosine in the chicken embryo (Maher and Pasquale, 1988).

In addition to being a component of focal adhesions, paxillin is also concentrated in the dense plaques of smooth muscle and the myotendinous junctions of skeletal muscle (Turner et al., 1991). These are both regions of actin–membrane interaction and homologous to the focal adhesions of cultured fibroblasts (Burridge et al., 1989). Transformation of cultured fibroblasts by RSV disrupts focal adhesions and their associated stress fibers (Ash et al., 1976; Edelman and Ya-

hara, 1976; Wang and Goldberg, 1976; David-Pfeuty and Singer, 1980). The phenotypic changes induced by RSV involve phosphorylation of proteins by the tyrosine kinase $pp60^{v\text{-}src}$, which has itself been localized to residual focal adhesions in transformed cells (Rohrschneider, 1980). Compared to other focal adhesion proteins, including talin (Pasquale et al., 1986; DeClue and Martin, 1987), vinculin (Sefton et al., 1981), and integrin (Hirst et al., 1986), paxillin is heavily phosphorylated on tyrosine residues in these cells (Glenney and Zokas, 1989) although the consequence of this phosphorylation remains to be determined. It seems unlikely that $pp60^{c\text{-}src}$ is the tyrosine kinase responsible for the phosphorylation of paxillin in the developing embryo since the tissues that express the highest levels of $pp60^{c\text{-}src}$, namely those of electrogenic origin, express some of the lowest levels of paxillin.

During the earlier stages of development cell–cell and cell–extracellular matrix junctions are continually remodeled to accommodate rapid cell proliferation, growth, and differentiation. In contrast, the same structures are relatively stable in adult tissues. As mentioned, it has been proposed that the normal cellular homologues of the oncogenes that give rise to uncontrolled cell division, cytoskeletal disruption, and decreased adhesion in transformed cells, may also coordinate the process of normal development via their tyrosine kinase activity (Adamson, 1987; Maher and Pasquale, 1988; Takata and Singer, 1988). In relation to this, immunolocalization studies have revealed that a high concentration of anti-phosphotyrosine labeling is seen close to the membrane at sites of cell–ECM and cell–cell junctions in embryonic tissues. This labeling is absent from similar locations in the adult tissues (Takata and Singer, 1988). Furthermore, while relatively high levels of protein phosphotyrosine have been reported at focal adhesions in normal early passage cells in culture (Maher et al., 1985), this signal is greatly attenuated in later passage cells when the cells are less motile and have developed a more highly organized cytoskeleton in the form of thicker stress fibers. Finding high levels of phosphotyrosine in focal adhesions also raises the possibility that cell adhesion per se stimulates tyrosine kinase activity both in culture and in the developing embryo. It is well documented that in the developing embryo cells respond to cues derived from interaction with their immediate ECM environment (Ekblom et al., 1986; Thiery et al., 1989). The transmembrane integrin molecules on the cell surface, responsible for the cells' interaction with the ECM (Hynes, 1987), are among the likely reporter molecules. The nature of the signals generated in response to adhesion to the ECM is unknown, but activation of membrane-bound tyrosine kinases and subsequent phosphorylation of substrates, like paxillin, that are located in close proximity to integrin molecules is one possibility. In this respect preliminary results indicate that in rat embryo fibroblasts selective tyrosine phosphorylation of paxillin is triggered by cell adhesion and cell spreading on fibronectin (Turner and Burridge, unpublished results).

In summary, paxillin has been identified as one of the major targets for tyrosine kinases in a variety of tissues during embryonic development. The change in the level of tyrosine phosphorylation of paxillin during the chick embryo development suggests that tyrosine phosphorylation of paxillin may regulate the formation of stable actin–membrane interactions required for normal organogenesis and adult organ function. The tyrosine-specific protein kinase responsible for the phosphorylation of paxillin during development remains to be identified, although the developmentally regulated tyrosine kinases identified by Pasquale and Singer (1989) are potential candidates.

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