Phosphotyrosine-modified Proteins Are Concentrated at the Membranes of Epithelial and Endothelial Cells during Tissue Development in Chick Embryos

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Abstract. We have used high affinity polyclonal antibodies specific for phosphotyrosine (PTyr) residues to examine the localization in various chick embryonic tissues in situ of PTyr-modified proteins by immunocytochemical methods. During the period from 9 to 21 d of development, most tissues exhibit elevated levels of PTyr-modified proteins as determined by immunoblotting experiments of tissue extracts with the anti-PTyr antibodies (Maher, P. A., and E. B. Pasquale. 1988. J. Cell Biol. 106:1747–1755). By immunofluorescence labeling of semithin frozen sections, the highest concentrations of PTyr immunolabeling in all of the embryonic tissues examined were localized to the membranes of the epithelial and endothelial cells with other cells showing no detectable labeling. These results were confirmed by immunoelectron microscopic labeling, which showed particularly high concentrations of PTyr-modified proteins close to the membranes at the apical junctions. The corresponding adult tissues showed no labeling. It is proposed that these results reflect the molecular basis for the functional plasticity of epithelial and endothelial cell junctions during embryonic development.

The phosphorylation of tyrosine residues of cellular proteins is thought to be important in cell transformation, growth, and differentiation. Tyrosine kinase activities are expressed by certain growth factor receptors and by the products of particular proto-oncogenes (for review see Hunter and Cooper, 1985). There is evidence that these and related activities may participate in regulating cell division and differentiation during embryonic development. Other investigators have focused on individual oncogenes, both those encoding tyrosine kinases and those not, and their expression in different tissues during embryogenesis (for reviews, see Muller and Verma, 1984; Adamson, 1987). In this and the preceding paper (Maher and Pasquale, 1988), we have instead obtained an overview of the pattern of tyrosine phosphorylation of proteins in the embryonic development of tissues. To achieve such an overview, we have used a recently described high affinity polyclonal antibody that is specific for phosphotyrosine (PTyr) residues to assay for PTyr modification. This antibody detects PTyr on a wide range of proteins including those in normal cell lines in culture (Wang, 1985; Maher et al., 1985). In the preceding paper (Maher and Pasquale, 1988), this anti-PTyr antibody was used to immunoblot extracts from a variety of chick embryonic tissues from 7 to 21 d of development. In this manner, it was shown that a discrete number of major PTyr-modified proteins was observed in all of the different tissues examined; these proteins were in the molecular mass range 60–220 kD. Their levels changed with the stage of development, becoming low or undetectable in most tissues in the adult chicken.

Here we extend these studies to the structural level, and use the anti-PTyr antibody in immunofluorescent and immunoelectron microscopic experiments to localize PTyr-modified proteins on frozen sections of intact fixed chick embryonic tissues. These immunocytochemical studies have revealed the intriguing fact that the highest concentrations of PTyr-modified proteins detected in all of the different tissues examined are localized to the epithelial and endothelial cells of the tissues, and in particular, at specific junctional regions of the plasma membranes of these cells. We suggest from these and other results that the high concentrations of PTyr-modified proteins at such membrane sites reflect an important functional plasticity of epithelial and endothelial cell junctions during embryonic development.

Materials and Methods

Immunolabeling Reagents

The anti-PTyr antibodies, prepared according to Wang (1985), were supplied by Dr. E. G. Pasquale and were the same as used in the accompanying paper (Maher and Pasquale, 1988). Rhodamine-conjugated affinity-purified
Figure 1. Localization of PTyr immunolabeling in the lung by immunofluorescence microscopy. Semithin frozen sections were indirectly labeled with the anti-PTyr antibodies (a, c, e, h, j, l, n, p, and r). Nomarski differential interference images of the corresponding fields are also shown (b, d, g, i, k, m, o, q, and s). (a and b) 9-d embryonic lung stained for PTyr. The basolateral aspect of the cell membrane and apical junctions (arrowheads) are stained for PTyr. The apical membrane (double arrows) is not labeled. (c and d) A survey view of 14-d embryonic lung stained for PTyr. The basolateral aspect of the cell membrane of the epithelium (Ep) is stained. Strong concentration of the label is seen in the apical junction of the epithelial cells (arrowhead). Endothelial cells of the blood vessel (Bv) are also positive for PTyr. Smooth muscle cells (M) surrounding the epithelium or the endothelium are not stained. Fibroblasts (e.g., F) scattered in the...
(Fab')2 fragments of goat anti-rabbit antibodies were obtained from Jackson Immuno Research Laboratories, Avondale, PA. N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-phallacidin was purchased from Molecular Probes Inc., Junction City, OR. Colloidal gold (5 nm)-conjugated affinity-purified guinea pig anti-rabbit antibodies were provided by Dr. G.A. Keller.

**Tissue Preparation and Cryosectioning**

Fertilized eggs of White Leghorn chickens were incubated at 38°C in a humidified chamber and embryos dissected on 9-20 d of incubation. Adult female chickens weighing 1-1.5 kg were used. Small pieces of tissue blocks were prepared from lung, small intestine, lens, and skeletal muscle of the three embryonic stages were fixed either in 3% paraformaldehyde in PBS (0.02 M sodium phosphate-0.15 M NaCl, pH 7.4) for 30 min at room temperature, or in periodate-lysine-paraformaldehyde fixative (PLP fixative) (McLean and Nakane, 1974) for 1-3 h at 4°C. Small blocks of cardiac tissues were prepared from the left ventricular wall after perfusion with either of the fixatives, and further fixed as described above. For immunofluorescence microscopy, both fixation procedures gave similar staining patterns. For immunoelectron microscopy, PLP fixed specimens were used because of the better preservation of the ultrastructure. After fixation, tissue blocks were transferred to PBS and stored in the refrigerator. Sections were cut and stained within 4 d after fixation, which gave consistent results. Some of the embryonic tissues were embedded in 2% polyacrylamide gel before cryosectioning to facilitate the preservation of the integrity of the tissue. Although the staining pattern was not affected by polyacrylamide gel embedding, it significantly reduced the intensity of the label. This procedure, therefore, was used only in some of the more delicate tissues, and the results were carefully compared with those of non-embedded specimens. Cryosectioning of the fixed tissues was carried out essentially according to the procedure of Tokuyasu (1980). Briefly, fixed tissue blocks were infused with 2.3 M sucrose-0.1 M sodium phosphate buffer, pH 7.4-0.02% sodium azide, mounted on a copper specimen holder and rapidly frozen in liquid nitrogen.

Semithin frozen sections (0.5-1.5-μm thick) were cut at -60°C with a Dupont-Sorvall ultramicrotome equipped with LTC-2 cryoattachment using glass knives. Sections were collected on glass slides. Ultrathin frozen sections (0.05-0.10-μm thick) were cut at -80°C in the same way, and collected on electron microscope grids coated with Formvar and carbon.

In some cases, pieces of tissues were fixed in 2% glutaraldehyde in PBS, osmicated, treated with tannic acid to enhance contrast, and embedded in Epon for conventional thin-section electron microscopy.

**Immunolabeling of Frozen Sections**

For immunofluorescence studies, semithin frozen sections attached on a glass slide were washed with 0.1 M glycine-PBS, and then blocked with 2% gelatin in PBS. After a rinse, sections were covered with the solution of anti-PTyr antibodies (10 μg/ml) for 30 min. Sections were rinsed and incubated with the rhodamine-conjugated F(ab')2 fragments of goat anti-rabbit IgG antibodies (10 μg/ml) for 30 min. After being washed, specimens were mounted in 90% glycerol-0.1 M Tris- HCl buffer, pH 8.5. For double labeling for PTyr and F-actin, NBD-phallacidin was diluted to 1:15, and used either before or after labeling with the anti-PTyr antibodies. Both procedures gave the same results. Specimens were observed with a Zeiss Photomicroscope III equipped with the epifluorescence system. Nomarski differential interference and immunofluorescence images were photographed.

For immunoelectron microscopy, specimens were washed with 0.1 M glycine-PBS and incubated with 1% gelatin-1% BSA-PBS for 15 min. After a brief rinse, sections were incubated with the anti-PTyr antibodies (10 μg/ml) for 45 min, washed, and then incubated with the colloidal gold-conjugated guinea pig anti-rabbit IgG antibodies for 45 min. After being washed with PBS, sections were fixed in 2% glutaraldehyde-0.1 M phosphate buffer, pH 7.4, and embedded in a mixture of methylcellulose, polyethylene glycol, and uranyl acetate using the adsorption staining method of Tokuyasu (1980). Specimens were observed in a Philips EM-300 electron microscope operated at 60 kV.

**Results**

**Localization of PTyr by Immunofluorescence Microscopy**

Semithin frozen sections of several chick embryonic tissues were examined by indirect immunofluorescence with the anti-PTyr antibody at developmental stages in which other studies from this laboratory (Maher and Pasquale, 1988) had indicated that high concentrations of PTyr-modified proteins were present.

In embryonic lung, the highest concentration of PTyr labeling was confined to the airway epithelium (Fig. 1, a, b, c, d) and the blood vessel endothelium (Fig. 1, c and d). Smooth muscle cells subtending the epithelium and surrounding the blood vessel endothelium did not become labeled, nor did fibroblasts that were scattered through the matrix. The immunofluorescent labeling with the anti-PTyr antibody throughout this paper, as was also the case in an earlier study (Maher et al., 1985), was entirely specific for PTyr residues. This is demonstrated for the embryonic lung in Fig. 1, j-m; the labeling of the epithelial cells was eliminated by an excess of free O-phospho-l-tyrosine (Fig. 1, j and k), but not by an equal concentration of free O-phospho-l-serine (Fig. 1, l and m) or O-phospho-l-threonine (not shown).

The PTyr labeling of the epithelial cells in both 9-d (Fig. 1 a) and 14-d (Fig. 1 c) lung was concentrated near the cell membranes, but was not uniformly distributed over them. In sections cut perpendicular to the epithelium, basolateral membranes were generally uniformly labeled; there was an enrichment of labeling at the apical junction (arrowheads in Fig. 1, a-d), whereas the apical membranes (double arrowheads in Fig. 1, a-d) exposed to the airway lumen (L) were not labeled. In appropriate sections cut parallel to the epithelium, PTyr labeling formed a continuous belt surrounding each epithelial cell at the level of the apical junction (Fig. 1, h and i). No labeling was detected in cellular organelles or in the cytoplasm generally.

Double fluorescent labeling for PTyr and for F-actin (with NBD-phallacidin) in the sections of 14-d lung (Fig. 1, e-g) showed a close correspondence of the two labels along the connective tissue matrix underneath the epithelium are not labeled. L, lumen. (e, f, and g) 14-d embryonic lung epithelium doubly labeled with the anti-PTyr antibodies (e), and with NBD-phallacidin (f). Colocalization of PTyr and F-actin is evident in the apical junctions as dots and short bars (e.g., arrowhead). (h and i) 14-d embryonic lung epithelium labeled for PTyr. Sections were cut nearly parallel to the surface of the epithelium. PTyr labeling is seen as a continuous belt surrounding the neck of each epithelial cell. (j and k) 14-d embryonic lung epithelium labeled with anti-PTyr antibodies in the presence of 10 mM O-phospho-l-tyrosine. Positive labeling in the basolateral cell membrane as shown in c and d is completely inhibited. (l and m) 14-d embryonic lung epithelium labeled with the anti-PTyr antibodies in the presence of 10 mM O-phospho-l-serine. Addition of O-phospho-l-serine has no effect on the staining for PTyr. Arrowhead shows the concentration of PTyr labeling in the apical junction of the epithelial cells. (n and o) A blood vessel in the 14-d embryonic lung stained for PTyr. Labeling for PTyr is seen in the endothelium lining the blood vessel. Concentration of PTyr labeling seems to be at the cell-to-cell junctions between endothelial cells (e.g., arrowhead). Red blood cells (arrow) in the lumen of the blood vessel are negative. (p and q) 19-d embryonic lung airway epithelium stained for PTyr. Only weak staining of the basolateral cell membrane is seen. (r and s) Adult lung airway epithelium stained for PTyr. No significant labeling is seen. Burs, 10 μm.
Figure 2. Localization of PTyr immunofluorescent labeling in the small intestine. Semithin frozen sections were indirectly immunolabeled with the anti-PTyr antibodies (a, c, and e). Nomarski differential interference images of the corresponding fields are also shown (b, d, and f). (a and b) 15-d embryonic intestine labeled for PTyr. Labeling for PTyr is seen in the basolateral aspect of the cell membrane of the epithelium (Ep). Endothelium of the blood vessels (e.g., arrows) are also positive for PTyr. Smooth muscle cells in the muscle coat (M) or the connective tissue cells (Ct) are negative. L, lumen. (c and d) 9-d embryonic intestinal epithelium stained for PTyr. Labeling for PTyr is seen along the basolateral cell membrane of the epithelium. Arrowhead shows the concentration of PTyr in the apical junction of the epithelial cells. (e and f) Adult intestinal epithelium stained for PTyr. No significant labeling is seen. The blood vessel endothelium (arrow) is not labeled either. Bar, 10 μm.

In the embryonic intestine, PTyr immunolabeling (Fig. 2) was again found to be largely confined to the epithelium and to the endothelium lining the blood vessels. A composite field of 15-d small intestine is shown in Fig. 2, a and b. The epithelial cell membranes showed intense labeling as did those of endothelial cells (arrows), but the connective tissue region (Ct) and the smooth muscle layer (M) were free of labeling. The 9-d intestine also showed epithelial cell mem-

epithelial cell membranes. In particular, both labels were concentrated at the apical junctions (arrowheads).

The PTyr labeling of the blood vessel endothelium of the 14-d lung (Fig. 1, n and o) was also nonuniform (arrowhead), but at the resolution of the light microscope, the endothelial cells were too thin to discern further structural details. A red blood cell in the vessel (Fig. 1, o, arrow) was not labeled.

By 19 d of development, the lung airway epithelium was only weakly and sporadically labeled for PTyr, mostly along the basolateral membrane (Fig. 1, p and g). In the adult lung, no labeling for PTyr was detected (Fig. 1, r and s).
brane localization of labeling (Fig. 2, c and d), with some apparent concentration at apical junctions (arrowheads). Adult intestine generally showed no PTyr labeling (Fig. 2, e and f).

Embryonic lens was examined because it is made up exclusively of specifically differentiated cells of epithelial origin. PTyr labeling was found on the cell membranes of the lens fibers throughout 9–20 d of development (Fig. 3), as well as at the membranes of the epithelium covering the mass of lens fibers (Fig. 3, a and b). In adult lens, on the other hand, no PTyr labeling was seen (Fig. 3, e and f).

In embryonic heart, the only structures clearly labeled for PTyr were endothelial cells lining blood vessels in both early stages (8 d) (Fig. 4, a and b) and late stages (20 d) (Fig. 4, c and d) of cardiac development. The striated muscle cells were not labeled. In particular, the intercalated disk regions of the muscle cells (arrows), which are structures analogous to the apical junctions of epithelial cells, remained unlabeled.
throughout development. In the adult, cardiac muscle endothelial cells showed no detectable PTyr labeling (Fig. 4, e and f).

High Resolution Localization of PTyr by Immuno Electron Microscopy

The light microscopic studies described above have limited resolution, and the precise localization of PTyr labeling in epithelial and endothelial cells could not be ascertained. For higher resolution, we immunolabeled ultrathin frozen sections with the anti-PTyr antibody followed by a colloidal gold-labeled secondary antibody. In Fig. 5 a is shown an apical junctional region of 14-d embryonic lung epithelium. Gold particles were distributed close to the membranes all around the edges of the apical junction. This localization was not observed in the presence of an excess of unlabeled PTyr. In contrast, the apical labeling was greatly reduced in the presence of unlabeled PTyr. We also observed an increase in the labeling of the lateral membranes in the presence of unlabeled PTyr. This suggests that PTyr is present in the lateral membranes of these cells.

Figure 5. Ultrastructural localization of PTyr in embryonic lung epithelium (a and b), and endothelium (c). Ultrathin frozen sections were stained for PTyr by the indirect immunogold method. (a) Apical junction region of the 14-d embryonic lung epithelium stained for PTyr. The label for PTyr is concentrated in the apical tight junction region along the cell membrane. Desmosome (D) is not labeled. L, lumen. (b) Apical junction region of the 14-d embryonic lung epithelium stained for PTyr in the presence of 10 mM O-phospho-L-tyrosine. Labeling for PTyr is inhibited. Also note the close appositions of the two cell membranes within the apical junction (arrows). L, lumen. (c) Endothelium of the blood vessel in 14-d embryonic lung stained for PTyr. Label is seen at the apical cell junction (arrow) and along the lateral cell membrane. L, lumen. Bars, 0.1 μm.
along the junctional region, but labeling stopped abruptly where the membrane-associated densities fell off sharply. Neither the desmosome nor the apical cell surface exposed to the lumen (L) was labeled. The specificity of the labeling obtained in Fig. 5a is demonstrated in Fig. 5b, which is of a similar field labeled with the same anti-PTyr antibody in the presence of 10 mM O-phospho-L-tyrosine. In Fig. 5b, the apical junctional region is delineated as a sequence of close appositions of the cell membrane (arrows, presumably tight junctions) with intervening membranes that are more separated (presumably adherens-type junctions). This is more clearly seen in plastic-embedded sections (not shown, but see Overton and Shoup [1964] and the freeze-fracture replicas of Talmon and Ben-Shaul [1979]).

In Fig. 5c is shown the immunogold labeling of a section of an endothelial cell lining a blood vessel in the 14-d lung. PTyr labeling was concentrated at the junctional region (arrow) and along the lateral membrane of the endothelial cell.

**Discussion**

Although tyrosine phosphorylation of proteins is considered to play an essential role in growth and differentiation, and the expression of a number of proto-oncogenes encoding tyrosine kinases has been demonstrated during embryogenesis (Muller and Verma, 1984; Hunter and Cooper, 1985), the specific proteins that are thus modified and the functions mediated by such modification are not well understood. Part of the difficulty is because the overall levels of PTyr in cells are always much smaller than those of phosphoserine and phosphothreonine. The analytical problem can in principle be overcome, however, by the use of antibodies that are specifically directed to PTyr and can distinguish it from phosphoserine and phosphothreonine. Many such anti-PTyr antibodies have been generated in different ways but that produced by Wang (1985) has several advantages for these studies (see Maher and Pasquale, 1988). We have now used this anti-PTyr antibody to localize the major PTyr-modified proteins in different tissues during chick embryonic development, both at the light and electron microscopic levels of resolution. Tissues from intact embryos were processed to produce semithin frozen sections for immunofluorescence labeling or ultrathin frozen sections for immunoelectron microscopy (Tokuyasu, 1980). With a wide range of different embryonic tissues, at different stages of development, remarkably similar results were obtained. High concentrations of anti-PTyr antibody labeling was found only in the epithelial and endothelial cells and only along their plasma membranes. Muscle cells (smooth or striated), fibroblasts and connective tissue, and other tissue cells showed no detectable labeling, although they generally constituted the largest part of the tissue.

In the epithelial cells, the membrane labeling was not uniform. It was not detected at the apical membranes (double arrowheads, Fig. 1, a and b), but was found along the basolateral membranes and was particularly intense at the apical cell–cell junctions. At the higher resolution of electron microscopy, the intense labeling at the apical cell–cell junctions of the epithelial cells was situated close to the membrane and confined to the region where the membrane was associated with an underlying density (Fig. 5a). This density very likely reflects the presence of membrane-associated actin microfilaments; at the light microscopic level, anti-PTyr antibody and actin labeling were largely codistributed (Fig. 1, e and f). The desmosomes were not labeled.

The endothelial cells lining the blood vessels of the several embryonic tissues also invariably showed a concentration of anti-PTyr antibody labeling along the lateral plasma membranes and at the apical cell–cell junctions, but not at the apical membranes facing the lumen of the vessel (Fig. 5c). The epithelial and endothelial cell labelings for PTyr were a property of embryonic tissues; the corresponding adult tissues were devoid of labeling.

These immunolabeling results do not imply that there are no significant amounts of PTyr-modified proteins elsewhere than at the membranes of the epithelial and endothelial cells in these embryonic tissues. Any PTyr-modified proteins that were uniformly distributed through the cytoplasm might not have been detected in our immunolabeling experiments. These considerations are relevant for a comparison of the present results with those in the companion paper (Maher and Pasquale, 1988), obtained by immunoblotting of extracts of the embryonic tissues with the same anti-PTyr antibody. In the latter studies, a considerable number of PTyr-modified protein bands were detected in each tissue. A number of the PTyr-containing proteins that give prominent bands in immunoblotting experiments appear to be readily extractable cytoplasmic proteins in the chicken embryonic tissues (Maher, P., and E. B. Pasquale, unpublished studies), and are therefore not likely to be the PTyr-modified proteins that we find localized to the epithelial and endothelial cell membranes. Some of the PTyr-modified protein bands appeared in the immunoblots to be homologous or identical to one another in different embryonic tissues; perhaps the PTyr-modified proteins that are responsible for the high intensity immunolabeling of the closely similar membrane sites of epithelial and endothelial cells in the different embryonic tissues are homologous to one another. Systematic studies of the sequential extraction of the tissues might provide useful information about these questions.

What is the significance of the high concentration of PTyr-modified proteins at the membranes of embryonic epithelia and endothelia? The property that characterizes epithelia and endothelia is that they both serve as barrier layers between a tissue and its environment; the barrier function is mediated by the apical junctions between neighboring epithelial or endothelial cells. It is these apical junctions that are intensely labeled with the anti-PTyr antibodies (Fig. 5a). That these apical junctions, but not other embryonic cell–cell junctions such as the intercalated disks between cardiac muscle cells (Fig. 4, a and b), are labeled suggests that the labeling is somehow connected with the barrier function of these junctions. On the other hand, that the epithelial and endothelial junctions exhibit high concentrations of PTyr-modified proteins only during embryonic development but not in the corresponding adult tissues implies that the PTyr-modification is associated with the rapid growth and expansion of the embryonic tissues. Overton and Shoup (1964) have shown, for example, that in early stages of embryonic development of the duodenal mucosa of the chick, a considerable number of mitoses occur throughout the epithelial layer, which means that the apical junctions must be continually remodeled to accommodate newly divided cells while continuing to main-
tain the epithelial barrier function. Correspondingly, early embryonic epithelia can be dissociated by trypsin into single cells, but in later embryonic mucosa and in the adult this is no longer possible (Overton and Shoup, 1964). The picture that emerges, therefore, is that PTyr modification of proteins associated with epithelial (and endothelial) junctions is a reflection of the special requirement that these junctions simultaneously retain their barrier integrity while exhibiting plasticity.

These considerations are consistent with other results that have been obtained with cells in culture. Maher et al. (1985), using the same anti-PTyr antibodies employed in this study, found that in a number of normal cell lines in subconfluent cultures the cell–cell and cell–substratum junctions were intensely immunofluorescently labeled. In these culture systems as well, the junctions are continually being made and broken down during cell division and cell migration, and must therefore exhibit plasticity.

By the nature of our antibody experiments, a composite of PTyr-modified proteins has been examined; it is therefore not known whether one or more tyrosine kinases are responsible for such modification in the different embryonic tissues, or even within a single type of epithelial or endothelial cell. A number of different oncogene products and hormone receptors possessing tyrosine kinase activity and their mRNAs have been detected in embryonic tissues (reviewed in Muller and Verma, 1984; Adamson, 1987); possibly only one particular kinase may be responsible for the high levels of PTyr modification of the proteins of the epithelial and endothelial junctions in the different embryonic tissues.

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