Intestinal Crypt Stem Cells Possess High Levels of Cytoskeletal-associated Phosphotyrosine-containing Proteins and Tyrosine Kinase Activity Relative to Differentiated Enterocytes

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Abstract. Growth and differentiation of stem cells is thought to be regulated by growth factors and responding protein tyrosine kinase activities. Comparing mitotic stem cells from the adult intestinal epithelium, isolated from the crypts of Lieberkuhn, with isolated differentiated absorptive cells we find major differences in the levels of phosphotyrosine-containing proteins. Crypt stem cells possess two major phosphotyrosine-containing polypeptides of 36 and 17 kD which have greater than 15 times more phosphotyrosine than that present in the polypeptides of differentiated enterocytes. Tyrosine kinase activity and similar phosphotyrosine-containing proteins are associated with the Triton cytoskeleton. Moreover, crypt tyrosine kinase(s) is active in vitro in phosphorylating similar cytoskeleton-associated substrates. These results suggest that cytoskeleton-associated phosphotyrosine kinase(s) and their substrates may play a role in growth and differentiation of adult intestinal epithelial cells.

The regulation of cell growth during development and differentiation is poorly understood but must include fine control over cell division. Some hints as to the regulatory proteins involved in growth control have come from recent analysis of viral transforming gene products and their normal cell homologues (Bishop, 1985; Hunter and Cooper, 1985; Adamson, 1987). The unregulated expression of these viral oncogenes, such as src, leads to uncontrolled cell growth suggesting that one function of the cellular protooncogenes is to play some role in growth control. Many of these genes code for protein tyrosine kinases. Tyrosine kinase activity has also been found to be a property of the membrane receptors for several growth factors and polypeptide hormones, including EGF, PDGF, insulin, and insulin-like growth factor, reinforcing the hypothesis that this class of protein kinases plays an important role in growth control (Frackelton et al., 1984; Ek and Heldin, 1984; Kasuga et al., 1982; Saski et al., 1985).

Protein tyrosine kinases and their substrates are often localized to regions of the plasma membrane enriched in the actin cytoskeleton (Landreth et al., 1985; Takata and Singer, 1988; Glenney and Zokas, 1989). Moreover, there is a correlation between transformation and the association of the transforming protein of Rous sarcoma virus, pp60src, with the cytoskeleton (Hamaguchi and Hanafusa, 1987). Recently, several studies have shown that tyrosine kinase activity is high in many normal embryonic tissues, including the intestinal epithelium, during periods of rapid proliferation (Mather and Pasquale, 1988; Takata and Singer, 1988).

The adult intestinal epithelium is a constantly differentiating system with a rapid turnover of cells (Weiser et al., 1986; Gordon, 1989). This simple epithelium is generated by a dividing stem cell population residing in the crypts of Lieberkuhn. In the adult, cells cease division while still in the crypts and differentiate as they migrate out of the crypts and reside in the basal one-third of the villi. After 2–3 d, the differentiated cells die and are extruded from the tip of the villus. Studies of the cells along the crypt to tip axis reveal a gradient of cells of differing states of differentiation exhibiting gradients of gene expression, as evidenced by the biochemical and morphological characteristics of the brush border (Gordon, 1989).

In the present work we use an antibody against phosphotyrosine to identify proteins that act as substrates for protein tyrosine kinases in cells isolated from different regions of the adult intestinal epithelium characterized by different degrees of differentiation. Western blot analysis demonstrates that undifferentiated crypt cells possess two predominant phosphotyrosine-containing proteins of 17 and 36 kD. The crypt cell population, containing all of the proliferating cells, exhibits a much higher level of phosphotyrosine in these proteins than was detected in the differentiated cells. Phosphotyrosine-containing forms of proteins of the same molecular mass are exclusively associated with the cytoskeleton and are phosphorylated exclusively on tyrosine in vitro. Our results demonstrate that the 36- and 17-kD polypeptides and the protein tyrosine kinase(s) which phosphorylate them are associated with the cytoskeleton. These findings suggest that one or more cytoskeletal-associated tyrosine kinases may play a role in growth control in this actively dividing adult stem cell population.
Methods and Materials

Cell Isolation

The proximal loop of chicken duodena was excised, slit open, thoroughly rinsed in ice cold saline, and stirred in phosphate-buffered sucrose solution at room temperature to release intact epithelial cells from the submucosa (Matsudaika and Burgess, 1979). Fractions containing cells sequentially isolated from the tip and basal third of the villus and from the crypt were obtained by a modification of the method of Weiser (1973) and Breimer et al. (1981). Briefly, pieces 1–2-cm long were stirred in a beaker for a designated length of time and transferred to a new beaker for the next time period. Cells from each fraction were taken after 11 (tip cells), 16, 29 (basal third cells), 39, and 64 (crypt cells) min. The purity of cell fractions was assayed by several means including determination of alkaline phosphatase by the method of Weiser (1973), and light microscopy.

Cytoskeletons were prepared by gently shaking cells for 30 min in 0.1% Triton X-400 in 75 mM KCl, 0.1 mM MgCl₂, 5 mM EGTA, 10 mM Tris, pH 6.9, containing 0.2 mM PMSF, 1 mg/liter aprotinin, and 1 mg/liter SBTI at room temperature. Cells were then washed by low speed centrifugation and resuspended in the above buffer lacking Triton.

Immunoprecipitation and Immunoblot Analysis

The methods for purification and characterization of the antibodies against phosphotyrosine have been described in detail elsewhere (Peaucellier et al., 1988). For immunoprecipitation experiments, crypt cytoskeletons were phosphorylated in vitro by incubation in a phosphorylation buffer containing 10 mM Hepes, 10 mM MnCl₂, 10 µM Na₂VO₄, 10 µg/ml Aprotinin, and 0.15% NP-40. The reaction was started by addition of [γ-³²P]ATP (435 Ci/mmol) to a final concentration of 50 µM and the samples were incubated at 25°C for 2 min. The [³²P]labeled proteins were solubilized in an immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris, 10 mM EDTA, 10 mM NaF, 10 µM Na₂VO₄, 5 mM PMSF, 1% Triton X-100, and 0.1 mM Aprotinin (Sigma Chemical Co., St. Louis, MO) and centrifuged at 100,000 g for 30 min at 4°C. The solubilized proteins were then incubated with affinity-purified antiphosphotyrosine antibody (0.3 µg/ml) at 4°C for 4 h at which 25 µl of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was added for each 0.1 µg of antibody. After 1 h, the immune complexes were collected by centrifugation, washed twice with immunoprecipitation buffer, once with 50 mM Tris, pH 7.5, solubilized in SDS gel sample buffer, and heated at 90°C for 5 min. The phosphoproteins were then analyzed by SDS micro slab PAGE, electroblotted onto Immobilon (Millipore/Continental Water Systems, Bedford, MA) membranes, and detected by autoradiography. For phosphoamino acid analysis, individual bands were cut out and hydrolyzed in 6 N HCl containing 50 µg each of phosphoserine, phosphothreonine, and phosphotyrosine at 110°C for 2 h. The hydrolysate was dried under vacuum and the phosphoamino acids were resolved by thin layer electrophoresis at pH 3.5.

Samples for Western immunoblot analysis were electrophoresed on SDS mini slab gels and electrotransferred to a sheet for 24 h in 0.45 µM Immobilon sheets. The sheets were then blocked in 10 mM Tris, pH 7.5, 0.2% NP-40, 1% gelatin, and 0.1% BSA for 12 h, after which they were incubated in blocking solution containing the affinity-purified antibody (0.3 µg/ml) for 4 h. After several washes in blocking buffer, the blots were incubated with [³²P]protein A 56 Ci/µg (ICN K & K Laboratories Inc., Plainview, NJ) at a concentration of 1 µCi/ml for 1 h. The blots were then washed in blocking buffer and dried.

Results

Characterization of Cell Fractions

The purity of each cell fraction was assayed in every preparation by phase-contrast microscopy which showed that the tip and crypt fractions were >95% pure (Fig. 1). The columnar tip cells possessed well-differentiated brush borders and were released in single cells to small sheets of cells (Fig. 1 A). The crypt fraction consisted of intact sacks of cells with the lumen internal, as in the intact tissue (Fig. 1 C). Crypt cells were cuboidal and were lacking in any distinct brush border. The mid fraction consisted of a few crypts mixed in with a majority of sheets of cells (Fig. 1 B). These sheets were composed of columnar cells, although not as tall as tip cells, which possessed brush borders with distinctly shorter microvilli than mature enterocytes. Using a brush border enzyme as a marker of the state of differentiation (Weiser, 1986), analysis of alkaline phosphatase activity from two separate cell preparations revealed that the tip cell fraction possessed 10-fold higher activity than did the crypt cells and about 4-fold higher than the mid cell fraction (tip = 24.27 U/mg; mid = 7.92 U/mg; crypt = 2.35 U/mg).

Phosphotyrosyl Proteins in Intact Cells

Samples of intact cells (equal amounts of protein) isolated from each region of the intestinal epithelium were run on 12% SDS-PAGE, electroblotted onto Immobilon, and probed for phosphotyrosyl-containing proteins with the antibody against phosphotyrosine. These Western blots revealed two major phosphotyrosyl-containing proteins with relative mobilities of 36 and 17 kD (Fig. 2, left). In addition, two polypeptides of ~50 and 45 kD were preferentially phosphorylated in crypt cells, and one very high molecular weight polypeptide was phosphorylated in crypt and basal villus cells, although at much lower levels than the 36- and 17-kD polypeptides. Interestingly, one protein of ~62-kD was found.
Table I. Relative Phosphotyrosine Content of 17- and 36-kD Proteins

|          | Tip | Mid | Crypt |
|----------|-----|-----|-------|
| 17-kD experiments | a   | 1.1 | 6.5   |
|           | b   | 1.2 | 15.5  |
|           | c   | 1.7 | 38.3  |
| 36-kD experiments | a   | 4.1 | 9.5   |
|           | b   | 0.3 | 16.4  |
|           | c   | 10.3| 29.1  |
|           | d   | 1.7 | 5.8   |

Cells isolated from the intestinal epithelium were analyzed by SDS-PAGE and blotted onto nitrocellulose. The blots were treated with the antibody to phosphotyrosine or with the antibody together with 5 mM phosphotyrosine as a competitive inhibitor. Antibody binding was detected by incubation with 125I-protein A followed by autoradiography. The phosphotyrosine content of the 17-kD (n = 3) and 36-kD (n = 4) proteins was estimated by scanning the autoradiographs and subtracting the values obtained in the presence of 5 mM phosphotyrosine from those obtained with the antibody alone. The values obtained from the tip fraction were set at 1.0 and the values from the other cell fractions are expressed relative to this value.

Figure 2. Detection of phosphotyrosine-containing proteins in the intestinal epithelium. Cells (100-μg protein) isolated from the tip of the villus (T), the basal third of the villus (M), and the crypt (C) were analyzed by SDS-PAGE and the proteins were blotted onto nitrocellulose. Phosphotyrosine-containing proteins were detected by incubating the blot with the antibody to phosphotyrosine. In one group of samples (right) 5 mM phosphotyrosine was included with the antibody to act as a competitive inhibitor. After washing, the bound antibody was detected with 125I-protein A and autoradiography. Two prominent bands of ~36 and 17 kD bound the antibody as seen in the left panel. However, phosphotyrosine completely inhibited antibody binding to these proteins (right) indicating that the antibody bound through an interaction with phosphotyrosine.

to be phosphorylated on a tyrosine only in the villus tip cells. The specificity of the antibody recognizing phosphotyrosine was confirmed by adding 5 mM free phosphotyrosine as a competitive inhibitor while the blot was incubating with the antibody (Fig. 2, right).

As seen in Fig. 2, a significant difference existed in the amount of phosphotyrosine-containing 36- and 17-kD polypeptides in cells of differing differentiated states. Significantly higher levels of phosphotyrosine associated with the 36- and 17-kD polypeptides were present in undifferentiated crypt cells than in either villus base or tip cells. Similarly, cells of the villus base possessed more phosphotyrosine in the 36- and 17-kD polypeptides than did villus tip cells. Quantitation of the relative levels of phosphotyrosine in the 36- and 17-kD polypeptides, by scanning autoradiographs from four separate experiments, revealed that crypt stem cells had on average 15 times more phosphotyrosine in 36-kD and an average of 20 times more phosphotyrosine in 17-kD than villus tip cells (Table I).

Cytoskeletal Association of Phosphotyrosyl Proteins

To determine whether these phosphotyrosine-containing proteins were associated with the cytoskeleton, equal amounts of protein from the Triton-soluble and -insoluble (cytoskeleton) fractions of isolated villus tip, villus basal third, and crypt cells were probed on a Western blot with the antibody against phosphotyrosine (Fig. 3). Our work and that by other investigators has shown that this cytoskeleton fractionation results in release of soluble proteins, dissolution of the plasma and internal membranes, and retention of the actin cytoskeleton, including the brush border cytoskeleton (Burgess, 1987). All phosphotyrosine-containing polypeptides were found associated with the detergent-insoluble cytoskeletal fraction. As in intact cells, the 36- and 17-kD polypeptides were the predominate phosphotyrosine-containing species. Several higher molecular mass phosphotyrosine-containing polypeptides were also detected. Moreover, as was found for intact cells, the level of phosphotyrosine-containing polypeptides was significantly higher in crypt cell cytoskeletons than in those of the basal third and tip of the villus.

In Vitro Tyrosine Phosphorylation of Crypt Cytoskeletons

Several attempts to detect in vivo phosphorylation of proteins in intact pieces of intestine by metabolic labeling proved unsuccessful because of poor uptake of phosphate, so we under-

Figure 3. Detection of phosphotyrosine-containing proteins in the cytoskeletal fraction of intestinal cells. Villus tip (T), basal third (M), and crypt cells (C) were isolated, the 0.1% Triton X-100-soluble (S) and insoluble (P) proteins precipitated with 10% TCA, and analyzed by SDS-PAGE. Phosphotyrosine containing proteins were detected by Western blot analysis using the antibody to phosphotyrosine as in Fig. 2. Samples on the right panel were incubated with antibody + 5 mM phosphotyrosine as a competitive inhibitor.
Figure 4. (A) In vitro phosphorylation of the cytoskeleton preparation from intestinal crypt cells. A sample (200 μg protein) of the detergent-insoluble cytoskeleton pellet was incubated in phosphorylation buffer containing 10 mM Hepes, pH 7.4, 10 mM MgCl₂, 10 μM Na₃VO₄, and 50 μM γ³²P-ATP (62.5 Ci/mmol) for 2 min at 25°C. The reaction was stopped by addition of 10% TCA and the precipitated proteins were washed with TCA, extracted with ether, and hydrolyzed in 6 N HCl at 110°C for 2 h. The samples were dried under vacuum, analyzed by two-dimensional high voltage electrophoresis at pH 1.9 (bottom to top) and at pH 3.5 (left to right), and then dried and subjected to autoradiography. The position of authentic standards included in the sample was detected by staining with ninhydrin. After autoradiography, the radioactive spots were cut out and counted in a scintillation counter: 43% of the counts were in phosphotyrosine, 41% in phosphoserine, and 16% in phosphothreonine. (B) Crypt cells were extracted with Triton X-100 and the detergent-insoluble cytoskeleton pellet was allowed to undergo phosphorylation in vitro with radioactive ATP as in A. The phosphorylated proteins were solubilized in immunoprecipitation buffer and incubated with the antibody to phosphotyrosine. The immune complexes were recovered by incubation with protein A-Sepharose and analyzed by SDS-PAGE. The phosphoproteins immunoprecipitated by this technique were detected by autoradiography of the gel. (C) Phosphorylated proteins immunoprecipitated as in A were resolved by SDS-PAGE and blotted onto Immobilon. The three predominant radioactive bands including the high molecular mass bands near the top of the gel (97-180 kD), the 36-kD band, and the 17-kD band, were localized by autoradiography, cut out, and hydrolyzed in 6 N HCl, and the phosphoamino acids analyzed by thin layer electrophoresis at pH 3.5. The position of standard phosphoamino acids was determined by staining with ninhydrin. 〇, origin.

took in vitro phosphorylation experiments with isolated crypt cell cytoskeletons. Isolated crypt cytoskeletons were incubated for a period of time with a phosphorylation buffer containing γ³²P-ATP. A fraction of the total in vitro-phosphorylated cytoskeletal proteins were hydrolyzed in HCl and analyzed by two-dimensional high voltage electrophoresis for phosphoamino acid content (Fig. 4 A). The autoradiograph of this analysis revealed a substantial amount of phosphotyrosine and phosphoserine with a small amount of phosphothreonine. The regions containing individual phosphoamino acids were cut out and counted in a scintillation counter. Of the total radioactivity incorporated into phosphoamino acids, 43% was in phosphotyrosine, 41% in phosphoserine, and 16% in phosphothreonine, clearly demonstrating that the cytoskeleton is highly enriched in tyrosine kinase activity.

To ascertain what proteins were phosphorylated on tyrosine in vitro, the radiolabeled cytoskeletal proteins were dissolved in immunoprecipitation buffer and the phosphotyrosine-containing proteins precipitated with antibody against phosphotyrosine. Analysis of the subsequent immunoprecipitated phosphorylated polypeptides by SDS-PAGE revealed two major phosphotyrosine-containing polypeptides of 17 and 36 kD (Fig. 4 B). However, the 36- and 17-kD polypeptides, while heavily phosphorylated, were not the only major phosphorylated polypeptides. Several other higher molecular mass polypeptides, ranging in molecular mass from 97 to 180 kD, were phosphorylated on tyrosine in vitro.

The phosphoamino acid composition of the ³²P-labeled proteins detected by analysis of immunoprecipitates, as in Fig. 4 B, was determined by blotting the gel onto Immobilon and cutting out the radioactive bands for partial acid hydrolysis and thin layer electrophoresis (Fig. 4 C). This analysis chemically confirmed that all phosphorylated polypeptides immunoprecipitated with the antibody against phosphotyrosine contained phosphotyrosine. Moreover, the 36-, 17-, and 97-180 kD (high molecular mass) polypeptides were phosphorylated only on tyrosine and not on serine or threonine.

Discussion

In this study we have demonstrated that undifferentiated mitotic stem cells in the intestinal epithelium are characterized by much higher levels of phosphotyrosine-containing proteins than are the differentiated cells located along the intestinal villus. Two cytoskeleton-associated polypeptides of 36 and 17 kD are the predominant protein tyrosine kinase substrates in the crypt cells. Moreover, tyrosine protein kinase activity is also associated with the cytoskeleton and is active in vitro.

Recent studies of developing embryonic chicken intestine
Our findings that crypt proteins phosphorylated on tyrosine are cytoskeleton-associated suggest that they may play some role in mediating some motile cellular event such as mitosis, cytokinesis, migration in these stem cells, or assembly of the brush border. Interestingly, Bretscher (1989) has recently shown that tyrosine phosphorylation of ezrin (p81) in A-431 cells correlates with major reorganizations of the actin cytoskeleton. Exactly how phosphorylation may affect these properties in vivo is unknown at this time. Further work will be necessary to determine the reason for the dramatic decrease in level of phosphotyrosine in proteins as enterocytes differentiate.

We have recently found that crypt cells possess 5-10 times higher in vitro protein tyrosine kinase activity of pp60^src than cells from the villus tip (Cartwright, C., W. Eckhart, S. Mamajewalla, and D. R. Burgess, manuscript in preparation). Since we find that one phosphotyrosine-containing protein in crypt cells is likely p36, calpactin I, our identification of pp60^src kinase activity is consistent with earlier reports that intestinal p36 is a good substrate in vitro for pp60^src (Glenney and Tack, 1985). On the other hand, since many cellular tyrosine kinases are plasma membrane-associated receptors for growth factors, our results do not rule out a role for growth factor receptor tyrosine kinase activity. Recently, several reports demonstrated that EGF promotes maturation, including the stimulation of DNA synthesis and the precocious appearance of specific digestive enzymes, of the intestinal epithelium in fetal and neonatal rodent intestines (for review see Lebenthal and Leung, 1987).

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