Protein Zero, a Nervous System Adhesion Molecule, Triggers Epithelial Reversion in Host Carcinoma Cells

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Abstract. Protein zero (P₀) is the immunoglobulin gene superfamily glycoprotein that mediates the self-adhesion of the Schwann cell plasma membrane that yields compact myelin. HeLa is a poorly differentiated carcinoma cell line that has lost characteristic morphological features of the cervical epithelium from which it originated. Normally, HeLa cells are not self-adherent. However, when P₀ is artificially expressed in this line, cells rapidly aggregate, and P₀ concentrates specifically at cell–cell contact sites. Rows of desmosomes are generated at these interfaces, the plasma membrane localization of cingulin and ZO-1, proteins that have been shown to be associated with tight junctions, is substantially increased, and cytokeratins coalesce into a cohesive intracellular network. Immunofluorescence patterns for the adherens junction proteins N-cadherin, a-catenin, and vinculin, and the desmosomal polypeptides desmoplakin, desmocollin, and desmoglein, are also markedly enhanced at the cell surface. Our data demonstrate that obligatory cell–cell adhesion, which in this case is initially brought about by the homophilic association of P₀ molecules across the intercellular cleft, triggers pronounced augmentation of the normally sluggish or sub-basal cell adhesion program in HeLa cells, culminating in suppression of the transformed state and reversion of the monolayer to an epithelioid phenotype. Furthermore, this response is apparently accompanied by an increase in mRNA and protein levels for desmoplakin and N-cadherin which are normally associated with epithelial junctions. Our conclusions are supported by analyses of ten proteins we examined immunochemically (P₀, cingulin, ZO-1, desmoplakin, desmoglein, desmocollin, N-cadherin, a-catenin, vinculin, and cytokeratin-18), and by quantitative polymerase chain reactions to measure relative amounts of desmoplakin and N-cadherin mRNAs. P₀ has no known signaling properties; the dramatic phenotypic changes we observed are highly likely to have developed in direct response to P₀-induced cell adhesion. More generally, the ability of this “foreign” membrane adhesion protein to stimulate desmosome and adherens junction formation by augmenting well-studied cadherin-based adhesion mechanisms raises the possibility that perhaps any bona fide cell adhesion molecule, when functionally expressed, can engage common intracellular pathways and trigger reversion of a carcinoma to an epithelial-like phenotype.

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The Journal of Cell Biology, Volume 131, Number 2, October 1995 465-482 465
tic and pleiomorphic, and may readily metastasize. It has become clear recently that in these transformed cell populations, the presence of functional CAMs, that are known to play roles in the development and maintenance of adhesion processes in normal epithelial monolayers, may be inversely correlated with the degree of metastatic potential of the corresponding tumor cells (Birchmeier et al., 1991; Frixen et al., 1991; Vleminkx et al., 1991; Takeichi, 1993).

For example, low levels of expression of E- and P-cadherin are found in carcinoma cell lines where extensive tumor invasiveness has been demonstrated (Frixen et al., 1991; Navarro et al., 1991; Vleminkx et al., 1991). From a therapeutic standpoint therefore, there is reason to believe that augmentation of functional Ca\(^{2+}\)-dependent CAMs in carcinoma cells may be one key to reengaging the normal epithelial cell program through suppression of the transformed state.

We have been studying the functional properties of P\(_o\), a transmembrane glycoprotein (Mr \(\sim 28,000\)) that mediates adhesion of Schwann cell plasma membrane surfaces to form compact myelin in the peripheral nervous system (PNS) of vertebrates. P\(_o\) is a member of the immunoglobulin (Ig) gene superfamily of CAMs that contains a single variable-like region in its extracellular segment (Lai et al., 1987; Lemke et al., 1988). This, and an analysis of its exon-intron structure, have led to the suggestion that P\(_o\) may possess adhesive properties similar to those of a primordial IgCAM from which all other members of this large superfamily may have arisen (Williams and Barclay, 1988).

According to this view, a general role for an ancestral P\(_o\)-like IgCAM might have been to contribute to intercellular adhesion, essential for the development of multicellular organisms (Williams and Barclay, 1988). A primordial P\(_o\)-like IgCAM was probably highly adhesive, and homophilic, and in certain tissues of some early organisms may have formed extraordinarily stable intercellular bonds. The fact that P\(_o\) expression in contemporary vertebrates is limited to myelinating cells may therefore not be surprising, since the compact myelin spiral, once formed, is not disassembled under normal conditions.

It has been demonstrated that after gene transfer P\(_o\) can mediate pronounced cell–cell adhesion in a Ca\(^{2+}\)-independent manner (D'Urso et al., 1990; Filbin et al., 1990; Schneider-Schaulies et al., 1990). We have shown that when the protein is expressed by cDNA transfection in HeLa cells, a human cervical carcinoma, it becomes concentrated at the lateral intercellular boundaries between adjacent P\(_o\)-expressing cells (D'Urso et al., 1990). By confocal immunofluorescent microscopy, it was clear that P\(_o\) expressed established very close, straight borders with each other. However, P\(_o\) remained uniformly distributed in the plasma membranes of those expresses that were isolated from one another on the coverslip, or in those that made contact with control nonexpressers (see Fig. 5, D'Urso et al., 1990). A preliminary ultrastructural examination of confluent P\(_o\) expresses suggested that at their plasma membrane appositions certain ultrastructural rearrangements took place. The loosely interdigitating finger-like processes observed in control cell populations were absent. Instead, a close (~5 nm), lengthy interface formed (D'Urso et al., 1990) that was reminiscent in its regular appearance and width of the intercellular gap of the intraperiod line of mature peripheral nerve myelin (Raine, 1984).

We interpreted these data as evidence for pronounced cell–cell adhesion induced by the homophilic association of the extracellular segments of P\(_o\) expressed on adjacent cell surfaces.

In this report, we show that HeLa cells generate a complex response to P\(_o\)-induced adhesion which greatly augments the placement of junctional proteins at cell–cell contacts, thereby compelling the assembly of certain junctional complexes, such as desmosomes and adherens junctions that are characteristic of normal epithelia. To our knowledge this is the first demonstration that a nervous system IgCAM, by obligating cells to adhere to one another, can trigger reversion of host tumor cells to an epitelioid phenotype. Our data raise the possibility that expression in carcinoma cells of any functional cell adhesion molecule that establishes close membrane apposition ultimately drives the cells to enhance the normal cell adhesion program such that stabilizing junctions are formed, and suppression of the transformed phenotype is achieved.

### Materials and Methods

#### Cell Culture and Transfection

HeLa cells, a cell line derived from a human cervical carcinoma, were routinely cultured in DMEM supplemented with 7.5 % FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all supplied by Gibco BRL, Gaithersburg, MD). To obtain clonal lines of P\(_o\) expresses, HeLa cells were transfected with a cDNA encoding the full-length P\(_o\) protein (Lemke and Axel, 1985). The P\(_o\)-cDNA was subcloned into the pECE vector (Ellis et al., 1986) and P\(_o\)ECE was introduced into the host HeLa cells along with the plasmid pSV2-neo (Southern and Berg, 1982) by liposome-mediated transfection as previously described (D'Urso et al., 1990). The day after transfection cells were split and seeded in complete DMEM containing 400 µg/ml of Geneticin G418 (Gibco) to select stable transformants. Cells transfected with the plasmid pSV2-neo only were used as control populations in all experiments. All stable transformants were maintained in culture in selection medium.

Unless otherwise noted, each experiment, control cells and P\(_o\) expresses were treated with 5 mM sodium butyrate, which has been shown to boost transcription of recombinant plasmids in mammalian cells (Gorman et al., 1983), and we have used extensively in previous work (D'Urso et al., 1990; Staugaitis et al., 1990; Allinquant et al., 1991). Butyrate treatment of nontransfected HeLa cells, and pSV2-neo-transfected cells did not induce epithelialization, as assessed morphologically or immunochromically with the markers we used in our studies.

#### Electron Microscopy

Stable P\(_o\) transformants (pSV2-neo and P\(_o\)ECE cotransfected) and control cells (pSV2-neo only) were plated and grown in multiwell dishes. 24-48 h after becoming confluent, both control and P\(_o\)-transfected cells were treated with 5 mM sodium butyrate for 16 h (Gorman et al., 1983), which increases the level of expression of P\(_o\) severalfold (see Fig. 1 C–E), after which they were washed with PBS, fixed with 2% paraformaldehyde and 2% glutaraldehyde in PBS, pH 7.2, for 1 h, postfix in 2% OsO\(_4\), and embedded in Epon before being processed for routine transmission electron microscopy. Ultrathin sections (0.6 µm) were cut perpendicular to the plane of the monolayer and contrasted with uranyl acetate and lead citrate.

#### Adhesion Assay

Confluent monolayers of P\(_o\) expresses and control cells were cultured for 16 h in complete DMEM containing 5 mM sodium butyrate. The presence or absence of butyrate in control cell populations was not found to affect cell aggregation (data not shown). After replacing the media with complete DMEM minus butyrate for at least 3–5 h, cells were trypsinized and
Antibodies

The assay was also performed in media containing 0.5 mM EGTA to reduce any residual Ca ++ under otherwise identical conditions.

pig desmosomal cadherin antibody (anti-desmocollin/desmoglein, Mechanic et al., 1991) and rabbit anti-desmoglein (Schmelz et al., 1986) were used, and Dr. S. Citi provided the rabbit cingulin antibody (Citi et al., 1988). The tight junction associated protein ZO-1 was detected using af-

Immunochemistry

Cells were plated on poly-L-lysine-coated coverslips, treated with bu-

Southern Blot Analysis

DNA amplified with the degenerate primers, using first strand cDNA synthesized in this way was used for polymerase chain reactions (PCR).

PCR Amplification

Specific PCR primers that recognize sequences in the carboxyl-terminal domain of human desmoplakin (hd1 and hd2) were designed based on the published sequence (Green et al., 1990). The primers were: hd1 (up-

RNA Isolation

Total RNA was prepared from confluent monolayers of controls and Po ex- pressors after overnight butyrate treatment. RNA isolation was per-

DNA was synthesized with (dT) 20 primers and the Moloney murine leukemia virus (M-MLV) H- Reverse Transcriptase (Superscript, Gibco Brl). Reverse transcription was allowed to proceed at 42°C for 1 h, followed by a 5 min incubation at 99°C to inactivate reverse transcriptase. First-strand cDNA

DNA was used for controls and Po expressors, cDNA was synthesized in this way was used for polymerase chain reactions (PCR).

PCR Amplification

Specific PCR primers that recognize sequences in the carboxyl-terminal domain of human desmoplakin (hd1 and hd2) were designed based on the published sequence (Green et al., 1990). The primers were: hd1 (up-

specifically described (D’Urso et al., 1990). The mouse monoclonal antibody we used recognized both desmoplakin I and II (Cowie et al., 1985). A guinea pig desmosomad cadherin antibody (anti-desmocollin/desmoglein, Me-

Mechanic et al., 1991) and rabbit anti-desmoglein (Schmelz et al., 1986) were used, and Dr. S. Citi provided the rabbit cingulin antibody (Citi et al., 1988). The tight junction associated protein ZO-1 was detected using af-

To determine if there was a difference in the relative levels of des-

To analyze the expression of mRNAs encoding molecules of the cad-

proximity of cDNAs added to each reaction mixture. These primers were: hgapdh1 (upstream): 5'-CTCAT- GACCAAGTCTAGCATTAC-3'; and hgapdh2 (downstream): 5'-TGCTG- TGCATAACCAGGAATAGACG-3'. Aliquots of reaction mixture were removed at various points between 10 and 20 cycles and assed for the presence and intensity of hgapdh bands on agarose gels.

RNA was used for controls and Po expressors, cDNA was synthesized in this way was used for polymerase chain reactions (PCR).

The assay was also performed in media containing 0.5 mM EGTA to che-

Dido reduced to single cell suspensions at a density of 10⁶ cells/ml. Cells suspensions were maintained with constant stirring (2 rpm/s) at 37°C in a humid-

measured by the guanidinium acid-phenol method (Chomczynski and Sac-

RNA concentration in each sample was determined by measur-

DNA amplified with the degenerate primers, using first strand cDNA

PCR experiments were performed in a Perkin-Elmer-Cetus Corp. (Norwalk, CT) DNA thermocycler. cDNA template was denatured at 94°C for 1 min and annealed with hd1 and hd2 primers at 50°C for 2 min, the temperature was then ramped over 1 min to 74°C and elongation by Taq DNA polymerase in the buffer recom-

Densitometric analysis of autoradiograms was performed on a Com-

We chose two highly conserved amino acid sequences by compar-

The EMBL accession numbers for the cDNAs we used are as follows: human desmoplakin, J05211; human GAPDH, M33197; and human N-cad-

amounted with 2.5-mM MgCl₂ proceeded for 3 min. Amplifications were performed under identical conditions using hgapdh primers. For all PCR reactions, 5-µl aliquots were removed at cycles 8, 10, 12, 14, 16, and 18, and 20. PCR products were visualized on 1% agarose gels. For autoradiogra-

hgapdh1 (upstream): 5'-CTCAT-GACCAAGTCTAGCATTAC-3'; and hgapdh2 (downstream): 5'-TGCGTGCATAACCAGGAATAGACG-3'. Aliquots of reaction mixture were removed at various points between 10 and 20 cycles and assessed for the presence and intensity of hgapdh bands on agarose gels.

To determine if there was a difference in the relative levels of des-

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Protein Preparation, SDS-PAGE, and Immunoblotting

Triton X-100 soluble and cytoskeletal fractions (Franke et al., 1981) of control and Pø-expressing HeLa cells were prepared as described. Briefly, cells were grown to confluence on 100-mm petri dishes, treated with 5 mM butyrate for 6-16 h, washed 5× with PBS, scraped off the dish, and pelleted (1,000 rpm, 5 min). Pellets were treated with 300 μl of lysis buffer (140 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM Tris [pH 7.4], leupeptin [0.5 μg/ml] and aprotinin [0.1 μmol/l]). After cell lysis, samples were centrifuged (15,000 g, 15 min), after which the supernatant was retained and the pellet resuspended in a high salt extraction buffer (lysis buffer with 1.5 mM KCl and 0.5% Triton X-100 added). Insoluble cytoskeletal fractions were pelleted from this suspension (15,000 g, 15 min), and solubilized (1% SDS, 10 mM Tris [pH 7.4], 5 mM EDTA). Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL), samples run on 7.5% SDS-PAGE, transferred to nitrocellulose, blocked with 5% milk protein, and incubated overnight with primary antibodies. After routine washing and secondary antibody incubations, blots were developed with the ECL chemiluminescence system (Biorad Labs., Hercules, CA).

Results

HeLa Cells Expressing Pø Become Polygonal and Exhibit Intracellular Concentration of Pø

HeLa cells were cotransfected with the vector pSV2neo (Southern and Berg, 1982) that encodes neomycin resistance, and a full-length rat Pø cDNA (Lemke and Axel, 1985) inserted into the vector pECE (Ellis et al., 1986) (PøECE). Permanent cell lines of Pø expressors and control cells (PSV2neo transformed only) were established by a selection in G418-containing medium. As detailed in Materials and Methods, control and Pø expressors were treated with butyrate upon reaching confluence. Under these conditions, permanent Pø-expressing cells displayed a markedly different morphology from the control HeLa cells (Fig. 1, A and B). Under phase-contrast microscopy, control HeLa cells (Fig. 1 A) appeared spindle shaped and fibroblastic, with clearly defined phase-bright cell-cell borders. By contrast, Pø-expressing cells (Fig. 1 B) were flat and polygonal, and their cell-cell boundaries harder to discern, suggesting that these cells made closer contact than control cells. The morphological differences observed by us by phase microscopy closely resemble those reported by Mege et al. (1988), when S180 cells, a mouse sarcoma (nonepithelial) line, were transfected with L-CAM, the chick homologue of E-cadherin. In this study, L-CAM apparently was capable of interacting with specific intracellular components of the S180 cells (e.g., actin) and it was concluded that cell-cell adhesion mediated by L-CAM directly brought about these cytoarchitectural changes.

When monolayers of permanent Pø expressors were examined using conventional immunofluorescence, without butyrate treatment, we observed a low level of Pø immunoreactivity (Fig. 1 C), and moreover, as expected, that 6 h-18 h treatment with butyrate (5 mM) enhanced Pø expression severalfold in these cells (Fig. 1 D). While low levels of Pø were detected in noninduced expressors in cell extracts (Fig. 1 E, [-] lane), substantially greater levels (at least 10-fold) were found in butyrate-treated cells (Fig. 1 E, [+] lane). Thus both immunofluorescence and protein blotting clearly demonstrate butyrate induction of Pø expression.

The conventional immunofluorescence images revealed that Pø became concentrated at regions of cell-cell contact (Fig. 1, C-D), as we have documented previously (D’Urso et al., 1990). In fact, by confocal microscopy (Fig. 2), markedly elevated concentrations of the protein at the lateral cell-cell contact sites were found. Analysis of Pø localization was facilitated by volume rendering of sequential images in the xy plane and subsequent sectioning in the xz plane (Fig. 2 B, panels 1-8). Using this approach, Pø localization was determined throughout representative cells in the monolayer. While some very low levels of Pø were found in the cytoplasm and at the free and basal cell surfaces, by far the highest levels of protein were found in regions where expressing cells made contact with each other (Fig. 2, panel 5, arrowheads). As shown to be the case in our previous study (D’Urso et al., 1990), and consonant with studies on cadherin localization in transfected cells (Nagafuchi et al., 1987; Hirano et al., 1987; Mege et al., 1988), this pattern of protein distribution has been generally accepted as morphological evidence for cell-cell adhesion.

Cell Surface Junctional Complexes Become Prominent in Pø-expressing HeLa Cells

Permanent cell lines expressing Pø and control cells were grown to confluence and processed for transmission electron microscopy. Ultrastructural analysis of confluent monolayers of control cell populations revealed that the plasma membranes of adjacent cells rarely formed recognizable contacts with each other. Usually, contact was limited to membrane interdigitations (Fig. 3 A), although occasional small desmosomes or adherens junctions could be identified between some cells (Fig. 3 B, arrow). In stable lines of Pø-expressing HeLa cells, however, evidence for close association between adjacent cells was commonly observed. In monolayers grown to confluence and maintained for 24 h, membrane protrusions were largely restricted to cell surfaces facing the medium, and for the most part were not present at the lateral intercellular borders. Instead, zones of close contact that exhibited electron-dense submembranous plaques and associated filaments were readily observed along these surfaces (Fig. 3 C, arrows). Between many cells, apical densities were noted (Fig. 3 C). These densities probably represented tight junctions, but frequently appeared “fused with”, and therefore could not be resolved from, adherens-type junctions (Fig. 3 C, arrows). In sections cut perpendicular to the plane of the coverslip so that the entire lateral domain could be sampled, these contacts were variable in length and in extent of development. Cells maintained for several hours longer (48 h) produced regular appositions along the entire lateral plasma membrane domain (Fig. 3, D and E). Electron dense plaques indicative of developing adherens junctions and desmosomes (Fig. 3 D, arrow, and 3, E and F) could be readily detected in these regions.

We concluded that Pø expressors form broad zones of close contact between each other. In addition, we inferred that these initial contacts and the ensuing development of junctional elements (Fig. 3, D-F), detected by electron mi-
Figure 1. $P_0$ expressors exhibit morphological changes and lateral localization of $P_0$ protein. Phase contrast images of confluent monolayers of (A) control PSV2 and (B) $P_0$-expressing HeLa cells. Whereas control HeLa tend to be rounded and spindle shaped with distinct boundaries and obvious intercellular distances, $P_0$ expressors are polygonal and cell–cell contacts appear close, almost fused in some cases. (C and D) Monolayers of permanent $P_0$ expressors labeled for $P_0$. Cells without butyrate treatment (C) have a low level of $P_0$ expression; addition of butyrate for 6–18 h substantially increases $P_0$ expression (D). In both cases, $P_0$ appears concentrated at lateral cell–cell contact sites. (E) Triton-soluble fractions of noninduced (−) and induced (+) $P_0$ expressors are run on 7.5% SDS-PAGE (200 μg/lane), transferred to nitrocellulose, and probed for $P_0$ using affinity purified antiserum. The noninduced cell fraction contains very little $P_0$ protein relative to the induced cell fraction. The (−) lane was exposed 5× longer (by chemiluminescence) than the (+) lane. Bar, 20 μm.

croscopy, was indicative of a complex cellular response to cell–cell adhesion that merited further investigation.

$P_0$ Expressors Rapidly Aggregate in Suspension

To directly determine whether $P_0$ was acting physiologically as an adhesion molecule in this system we employed a classic adhesion assay (Brackenbury et al., 1977) that measures the capacity of single cells to aggregate over time when suspended at low density in Ca++ free medium.

HeLa cells grown to confluence and butyrate treated were used in this assay. In stable lines of $P_0$, transformants the polypeptide can be readily detected by indirect immunofluorescence at the lateral borders between cells in the monolayer (Fig. 4 A). Mild trypsinization yielded >98% single cell suspensions of control (not shown) and $P_0$-expressing cell populations (Fig. 4 B) that were washed free of protease and maintained at low density (10⁶ cells/ml) in Ca++ free medium with constant stirring. Although intracellular $P_0$ glycoprotein was apparent by immunofluorescence in the trypsinized expressor cells (Fig. 4 B), as expected, protease treatment rendered the molecule undetectable at the cell surface. Within 15 min of incubation, however, small aggregates consisting of 2–4 cells were readily observed by phase microscopy in the $P_0$-expressing population, and on immunostaining, intense $P_0$ immunofluorescence was detected at cell surfaces and was markedly concentrated at cell–cell interfaces (Fig. 4 C). Clearly, the internal pool of $P_0$ that was protected from trypsinization, and/or newly synthesized $P_0$ had reached the cell surface during this short time interval, where it homophilically mediated the development of cell aggregates. During the course of the experiments, the cellular aggregates in the $P_0$, expressing population became quite large (Fig. 4 D), and the number of cells participating in aggregates was always far greater than in the control cell population (Fig. 4 E). For example, after 210 min of incubation, 55% of the $P_0$ expressors were in aggregates, as compared with 22% of the controls.

These experiments show that once $P_0$ reaches the HeLa cell surface, expressors begin to aggregate within minutes. The fact that $P_0$ is found highly concentrated at the sites of cell–cell contact in the aggregates, and that cell aggregation can occur under Ca++ free conditions, argues that $P_0$...
is the initiator of cell adhesion in this system, and that other known adhesion molecules such as members of the cadherin (Damsky et al., 1983; Ozawa et al., 1989; Takeichi, 1991) and integrin families (Albelda and Buck, 1990; Hynes, 1992) are not initially involved.

**Tight Junctional and Desmosomal-associated Proteins Localize to Intercellular Boundary Regions between P₀ Expressors**

Ultrastructural analysis of P₀-expressing cells (Fig. 3) indicated that a remarkable response to P₀-induced adhesion that developed over time across extensive areas of the monolayer was the formation of junctional complexes. To begin to study the physiological parameters of this response, we examined eight known representative polypeptide constituents of these junctions.

We used antibodies against cingulin and ZO-1, proteins which are believed to be associated with tight junctions (Stevenson et al., 1986; Anderson et al., 1988; Citi et al., 1988), and desmoplakin, desmocollin, and desmoglein, (Cowin and Garrod, 1983; Cowin et al., 1985; Schmelz et al., 1986; Green et al., 1990) polypeptide components of desmosomes, to map the distribution of these structures across monolayers of P₀ expressors and control cells grown in normal (i.e., calcium-containing) medium.

Cingulin is a cytoplasmic protein restricted to the subapical region of epithelial cells where tight junctions are found (Citi et al., 1989). In control cell populations of pSV2-neo transformants, cingulin immunoreactivity was distributed in a discontinuous, dot-like pattern. Rarely, short linear stretches of immunofluorescence were detected (Fig. 5 A, arrows) but for the most part, no clearly defined organization was discernible. In contrast, P₀ expressors exhibited strong immunofluorescence selectively localized at the subapical regions of cells that were in contact with each other. Cingulin was usually distributed in what appeared to be a thin, continuous belt (Fig. 5 B) that under high magnification could often be resolved into parallel belts (Fig. 5 C, arrows), each of which probably local-
ized under adjacent cell membranes. Another cytoplasmic protein found associated with tight junctions, as well as at cell–cell contacts of certain nonepithelial cell types, (Anderson et al., 1988; Stevenson et al., 1986; Howarth et al., 1992, 1994), is ZO-1. This protein showed a pattern of distribution similar to cingulin; in control cells (Fig. 5 D), ZO-1 was occasionally found at cell–cell contact sites, whereas ZO-1 immunofluorescence accumulated quite extensively at the membranes of \( P_o \)-expressing cells (Fig. 5 E). The distribution of these tight junction–associated proteins and \( P_o \) did not overlap; cingulin and ZO-1 were always detected in a narrow band above (i.e., “apical” to) the uppermost level of \( P_o \) reactivity and so the position of the cingulin and ZO-1 belts denotes the boundary below which \( P_o \) concentrates, and other junctional structures form (see below).

The aggregation experiments, taken together with the timed ultrastructural studies, suggested that adhesion mediated by \( P_o \) was a prerequisite for subsequent junction formation. We therefore directly examined the time course of appearance of desmosomes relative to the development of high levels of \( P_o \) fluorescence along intercellular borders. Cells were grown in medium containing \( Ca^{++} \), which is required for desmosome assembly (Hennings and Holbrook, 1983; Mattey and Garrod, 1986; Mattey et al., 1990), and after fixation, processed for immunofluorescence with \( P_o \) and desmoplakin antibodies.

The desmoplakins are a set of closely related polypeptides (\( M_r \sim 215,000-250,000 \)) that are found in association with the desmosomal plaque, where they may function in anchoring intermediate filaments to the plasma membrane (Cowin et al., 1985; Schwarz et al., 1990; Stappenbeck and Green, 1992; Garrod, 1993). Desmoplakin immunoreactivity is maximally visualized when cells or tissues are pretreated with 100% methanol; however, this agent was found to extract \( P_o \) from cell membranes. Although \( P_o \) im-

Figure 3. Development of junctional specializations in \( P_o \) transformants. Transmission electron micrographs of confluent, sodium butyrate-treated monolayers of (A and B) control cells and (C, D, E, and F) stable \( P_o \) transformants. At the borders of control cells interdigitating microvilli (A) and rarely, small desmosomes (B) can be observed. In contrast, within 24 h postbutyrate treatment, (C) adjacent plasma membranes of \( P_o \) expressors begin to develop apical subplasmalemmal densities (arrows). By 48 h, lateral domains display broad appositions (D and E) adherens junctions, and desmosomes. Desmosomes are clearly visible (D, arrows, and F). Bars: (A) 0.5 \( \mu \)m; (B–F) 0.1 \( \mu \)m.
munostaining in these cells is also greatly reduced when fixed with 1% paraformaldehyde, this milder fixation did allow us to localize both P0 and desmoplakin on the same coverslip. At early time points postbutyrate treatment, P0 immunofluorescence was detectable, but desmoplakin immunoreactivity was not apparent until several hours later. In fact, in many instances, P0 was detected completely concentrated in a continuous "island" or zone along entire intercellular boundaries (Fig. 6, A and D). Desmoplakin, by contrast, was distributed in regular dot-like arrays, presumably representing individual desmosomes in the process of formation, in discrete regions within the P0 adhesion island (Fig. 6, B and C). Eventually, rows of desmoplakin fluorescence, frequently double rows, developed within the P0 adhesive zone (Fig. 6, E and F).

The striking contrast between desmoplakin immunofluorescence detectable at the cell surface in the P0 expressors as compared with control cells is dramatically demonstrated by confocal microscopy (Fig. 6, G and H). In confluent control cell populations (Fig. 6 G), occasional short rows of isolated desmoplakin reactivity are identified (arrows). This is expected, since by thin-section electron microscopy, isolated fully developed desmosomes were occasionally observed between these cells (Fig. 3 B). Desmoplakin immunofluorescence of the confluent P0 expressors (Fig. 6 H) is so pronounced however that individual cell outlines can be readily appreciated. Immunofluorescence for desmocollin and desmoglein (Fig. 7), integral membrane proteins involved in the adhesive interaction of desmosomes (Goodwin et al., 1990; Holton et al., 1990; Koch et al., 1990; Cowin and Brown, 1993; Garrod, 1993), exhibited a dramatically increased distribution at the lateral cell membranes of P0 expressors. The immunofluorescence for desmosomal cadherins was particularly impressive, in that it outlined cell perimeters (Fig. 7 D). Taken together, these results argue that P0-mediated adhesion leads to increased desmosome formation in HeLa cells.

The Adherens Junction-related Proteins
N-Cadherin, α-Catenin, and Vinculin, Become Markedly Enhanced and Organized at Cell–Cell Contact Sites of P0 Expressors

In normal epithelia, adherens junctions may be found immediately subjacent to the apical tight junctions. A number of polypeptides involved in adherens junction formation have been identified (Geiger and Ayalon, 1992; Tsukita et al., 1992), including cadherins (Boller et al., 1985; Volk and Geiger, 1986a,b), catenins (Herrenknecht et al., 1991; Nagafuchi et al., 1991), vinculin (Geiger, 1979; Tsukita et al., 1992), and ZO-1 (Itoh et al., 1993, and see above). Cadherins may play "instructive" roles in the establishment of cell–cell adhesion (Doherty et al., 1991), and it has been suggested that E-cadherin expression is a prerequisite for junction formation (Gumbiner et al., 1988). Both N- and E-cadherins have been identified in adherens junctions (Boller et al., 1985; Volk and Geiger, 1986a,b; and see Geiger et al., 1987), and are distributed as well across nonjunctional plasma membrane surfaces; normally, however, HeLa cells express very low levels of N-, but not E-cadherin (Ozawa et al., 1989; Herrenknecht et al., 1991; Knudsen, K. A., personal communication, and this study, see below). We therefore asked whether the organization of N-cadherin into adherens junctions might be affected by P0-initiated cell adhesion.

N-cadherin antiserum (Volk et al., 1990) prominently labeled plasma membrane surfaces of expressors (Fig. 8 B), but not of control cells (Fig. 8 A). The immunofluorescence was extensively concentrated at lateral cell–cell boundaries in a continuous pattern, although puncta of fluorescence, probably corresponding to adherens junctions, were...
Figure 5. The tight junction–associated proteins cingulin and ZO-1 become highly organized at the cell surface. Cingulin immunofluorescence of (A) control PSV2-neo transformants and (B and C) Po expressors. (A) In control cell populations, cingulin is sparsely detected along apical plasma membrane segments (arrows), but is distributed along the entire apical cell perimeter of Po expressors (B), where at higher magnification, it may sometimes be resolved into two parallel belts (C, arrows). Likewise, while small stretches of control PSV2 cells are ZO-1 positive (D), Po-expressing cells demonstrate extensive ZO-1 labeling at apical cell contact sites (E). Bars: (A, B, D, and E) 20 μm; (C) 2 μm.

plainly visualized. When specific antiserum raised against E-cadherin (Fannon et al., 1995) was used, no labeling in either control or Po expressors was detected (not shown).

α-Catenin, a member of a family of cytoplasmic molecules which are found in association with the classic cadherins (Ozawa et al., 1989; Herrenknecht et al., 1991; Hinck et al., 1994; Nagafuchi et al., 1994; Nathke et al., 1994; Watabe et al., 1994), has been found experimentally to be important for junction formation. It has been postulated that the set of catenins, perhaps in coordination with certain unrelated cytoplasmic polypeptides (Ozawa et al., 1989; Cowin and Brown, 1993; Gumbiner, 1993; Hinck et al., 1994; Nagafuchi et al., 1994), regulate intercellular adhesion by mediating linkage of cadherins with the cytoskeleton. The Po expressors were found to have markedly enhanced amounts of α-catenin (Fig. 8 D) at the cell surface, as compared with the controls (Fig. 8 C).

Vinculin is a cytoplasmic protein associated with adherens junctions and focal contacts whose role, like α-catenin, may possibly be to link adherens junction associated cadherins to the cytoskeleton (Geiger, 1979; Geiger et al., 1987; Geiger and Ayalon, 1992; Tsukita et al., 1992). Vinculin was found in focal contacts at the substrate level of both control and Po expressors (not shown). However, in agreement with its putative role in cadherin adhesion, and with our results above, no vinculin labeling was found when focusing above the substrate in control cells (Fig. 8 E), but vinculin was clearly organized at the lateral borders of Po expressors (Fig. 8 F) above the substrate.

Desmoplakin, N-Cadherin, and α-Catenin Are Relatively Increased in Response to Po-mediated Cell Adhesion

The marked increase of desmoplakin immunofluorescence at the plasma membrane of confluent Po expressors could
Figure 6. Desmoplakin and P₀ colocalize at adhesive cellular interfaces. (A–F) P₀ expressors on coverslips were simultaneously labeled with P₀ and desmoplakin antibodies. At short time intervals (within 24 h; A, B, and C), P₀ is found concentrated between neighboring cells. In the same cells, desmoplakin is in linear arrays that are not yet completely continuous. In B, the arrows indicate a plasma membrane segment that at all levels of focus contains P₀ (see A) but is devoid of desmoplakin. In C, both P₀ and desmoplakin are revealed. At later time points (up to 48 h), linear bands of desmosomes are revealed by desmoplakin immunofluorescence (E and F). By confocal microscopy (G), control cells exhibit little desmoplakin at the cell surface (arrow). By contrast, P₀ transformants express so much desmoplakin that cell outlines are readily discerned (H). Bars: (A–F) 2 μm; (G and H) 10 μm.
be the result of stabilization of the protein at the surface, an increase in the transcription rate of the desmoplakin gene, and/or stabilization of the desmoplakin mRNA. According to the current literature, it is generally believed that the formation of desmosomes between cells is accompanied by a reduction in the rate of proteolytic breakdown of a relatively constant pool of soluble desmosomal polypeptide components, including desmoplakin (Pasdar and Nelson, 1988a,b; Pasdar et al., 1991). If this were so, it might be expected that equivalent relative mass amounts of desmoplakin mRNAs would be observed in control and expressing cell populations. If, on the other hand, the appearance of desmoplakin at high density at the cell surfaces of P₀ expressors was the result of an increase in gene transcription or a stabilization of desmoplakin mRNAs, then an increase in the relative levels of these specific mRNAs would be detected in the confluent expressors.

To begin to address this in a preliminary way, we synthesized first-strand cDNA from total RNA prepared from confluent control cells or P₀ expressors. Equivalent amounts of control and P₀ expressor cDNA (~10 ng/reaction) were then used as templates in PCR reactions containing human desmoplakin specific oligonucleotide primers, and [α³²P]dATP as a source of label for quantitation. To ensure that equivalent amounts of control and expressor cDNA template were used in these experiments, some reactions were primed with oligonucleotides specific for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a gene whose mRNA levels have been shown to be constant under a variety of conditions, and therefore has become useful in standardizing PCR reactions (not shown, but see Materials and Methods).

In these experiments, autoradiographs of agarose gels in which aliquots from several cycles (cycles 8–30, were routinely sampled) were displayed revealed, as expected, single bands of 1,682 bp, corresponding to the predicted length of the segment flanked by the desmoplakin-specific primers (Fig. 9 A, lanes 1–4). However, the intensities of those bands obtained in the reactions containing cDNA prepared from the P₀ expressors were visibly stronger in the earlier cycles when the PCR reaction is not rate limited by reagent depletion (Fig. 9 A, lanes 2 and 4) than in the control reaction mixture (Fig. 9 A, lanes 1 and 3). Densitometric scanning of autoradiographs derived from five different sets of cDNA preparations used for these studies demonstrated at least 6-, and up to 10-fold as much label.
Figure 8. N-cadherin, α-catenin, and vinculin become localized to lateral cell–cell contact sites in P$_e$ expressors. (A–F) Control and P$_e$-expressing HeLa cells were plated on coverslips and prepared for immunohistochemistry after reaching confluency. Unlike control cells (A), cells expressing P$_e$ (B) were found to have substantial amounts of N-cadherin concentrated at lateral zones of cell–cell contact. Moreover, the cadherin-associated protein, α-catenin, was not detected in control HeLa cells (C), but was readily found at regions of membrane contact of P$_e$-expressing cells (D). Vinculin did not localize to apical cell contact sites of PSV2 cells (E), but did in P$_e$-expressing cells (F). These adherens junction-associated proteins were found to localize at the cell perimeters where expressing cells make contact, as did P$_e$, the desmosomal proteins, and the tight junction-associated proteins. Bar, 20 μm.
incorporation in the Po expression reaction mixture than in the control. It is likely, therefore, that an upregulation of desmoplakin mRNA transcription, a stabilization of these mRNAs, or both, occurs in the Po expression population. Whatever the mechanism, the protein levels of desmoplakin are correspondingly increased in immunoblots of Po expressors (Fig. 9 A, lane 6) as compared to PSV2 control cells (Fig. 9 B, lane 5). Additional work should elucidate the subcellular basis for the increased mRNA and protein levels of this desmosomal component.

Given the increase in N-cadherin fluorescence at the surface of the expressors, we also looked for a relative change in mRNA levels for N-cadherin in the Po expressors, as compared with HeLa cell controls. We synthesized degenerate primers against highly homologous putative Ca++-binding domains common to N- and E-cadherin (Hatta et al., 1988) (see Materials and Methods) which were shown to recognize authentic N- and E-cadherin with control template encoding human N- or E-cadherin. As expected from our immunocytochemical data (see above, and Fig. 8, A–B), as well as previous work (Ozawa et al., 1989; Herrenknecht et al., 1991; Knudsen, K. A., personal communication) only N-cadherin could be detected when normal Hela cell cDNA was used as a template. These primers were used in PCR reactions containing Po expressors or HeLa cell cDNA (as above) except isotopic label was omitted. After electrophoresis of the reaction products obtained on completion of cycle 25, gels were incubated in ethidium bromide (Fig. 9 B, lanes 1 and 2), photographed, and processed for DNA blotting using an α-32P-labeled human N-cadherin cDNA we isolated as the probe. The ethidium-stained preparations revealed a single band of the predicted size for human N-cadherin in the reaction mixtures containing cDNA from the Po expressors (Fig. 9 B, lane 1), but not in reactions from the control cells (Fig. 9 B, lane 2) or when no cDNA template was added (not shown). DNA blots probed with N-cadherin cDNA, autoradiographed and densitometrically scanned revealed at least 10-fold more isotopic label in the Po expressors (Fig. 9 B, lane 4) than in control cells (Fig. 9 B, lane 5). Molecular cloning of the PCR-amplified DNA from control or Po expressor reaction mixtures yielded 21 individual colonies, all of which were found by nucleotide sequence analysis to contain plasmids with inserts encoding human N-cadherin exclusively.

Since RT-PCR analysis demonstrated an increase in N-cadherin mRNA in Po expressors, extracts of PSV2 cells and Po-expressing cells were subjected to Western blot analysis using monoclonal anti-N-cadherin (Fig. 9 C). Corroborating our immunocytochemical data (Fig. 8 B), N-cadherin was enriched in Po expressors (Fig. 9 C, Po). Moreover, there is clearly an increase in N-cadherin protein (Fig. 9 C, Po, [+] after butyrate treatment increases Po expression (Fig. 1, C–E), when compared to non-butyrate-treated cells (Fig. 9 C, Po, [−]), when there is only a low level of Po protein in these cells (Fig. 1, C–E). In control PSV2 cells, however, N-cadherin was not detected (Fig. 9 C, PSV2), either without (−) or with (+) butyrate treatment. The fact that there is an increase in N-cadherin protein levels after an increase in Po protein (see Fig. 1 E), while there is no change in control cells with butyrate treatment, argues strongly that the expression of Po brings about the increase in N-cadherin found in these cells.

In addition to desmoplakin and N-cadherin, two other proteins, ZO-1 and α-catenin, were detected using immunoblot techniques. Interestingly, ZO-1 protein levels were the same in control and Po-expressing HeLa cells, with or without butyrate treatment (Fig. 9 D), suggesting that the immunocytochemical data (see Fig. 5, D–E) reflects a reorganization of existing ZO-1 to cell–cell contact regions. These results also indicate that butyrate treatment specifically stimulates the expression of the PoECE vector in these cells. Moreover, the ZO-1 immunoblots can serve as an internal standard for the other blots of the cytoskeletal fraction, including desmoplakin (Fig. 9 A, lanes 5–6) and α-catenin (Fig. 9 E). Like desmoplakin and N-cadherin, α-catenin is substantially increased in Po expressors when compared to PSV2 cells (Fig. 9 E). These cells therefore appear to show a selective response to Po-mediated adhesion, the exact nature of which requires further study.
Reorganization of the Keratin Filament Network Occurs in P₀ Expressors

A prominent intracellular feature of mature desmosomes are the bundles of intermediate filaments that are found associated with the plaque region (Farquhar and Palade, 1963; Schwarz et al., 1990; Stappenbeck and Green, 1992; Garrod et al., 1993; Stappenbeck et al., 1993). These filaments, composed of organized keratins, are readily observed by transmission electron microscopy, and extend deeply into the cytoplasm on either side of the plasma membrane. Ultrastructurally, we had noted in confluent P₀ expressors that shortly after butyrate treatment, plasma membrane thickenings denoting developing desmosomes were clearly apparent (see Fig. 3), but intermediate filament assembly at these structures seemed delayed for several hours. To assess the development of the keratin network, we performed immunohistochemistry on the cultured cells, treating coverslips with antibodies against both desmoplakin (Cowin et al., 1985) and cytokeratin-18 (Ramaekers et al., 1983), known to be expressed in HeLa cells (Moll et al., 1982). In control cell populations (Fig. 10 A) keratin filaments frequently appeared elongated and sparsely distributed. In the P₀ expressors, however (Fig. 10 B), keratin bundles were conspicuous and perinuclear, and in many cases, double labeling with anti-desmoplakin (Fig. 10 C) showed that the filament bundles were aligned from one cell to another. In fact, cytokeratin bundles frequently were inserted into the surface desmosomes (Fig. 10 C, arrows). Slot-blots of total cell lysates from postbutyrate-treated control and P₀ expressors probed with keratin antisera revealed virtually equivalent staining intensities in the two populations (not shown). These results indicate that the increase in desmosome formation found in P₀ expressors leads to the formation of an intercellular network of keratin filaments from an apparently constant keratin pool.

Discussion

It is generally accepted that in forming epithelia, surface interactions between adhesion molecules in the cadherin superfamily are the starting point for the molecular cascade that ultimately leads to the formation of the differentiated phenotype (Geiger and Ayalon, 1992; McNeil et al., 1993; Takeichi, 1991; Wheelock and Jensen, 1992, Watabe et al., 1994). Thereafter, the maintenance of the normal epithelial phenotype requires constant cell–cell contact that is sustained by adhesion molecules expressed on cell surfaces. In many self-adherent epithelial cells the first step in the adhesion process is mediated by uvomorulin (E-cadherin), and it has been proposed that E-cadherin may in many systems exert a hierarchical control over the subsequent formation of junctional elements (Gumbiner et al., 1988; McNeil et al., 1993). HeLa cells however are an unusual epithelial derivative in that although they express cytokeratins (Moll et al., 1982), they do not synthesize detectable levels of E-cadherin, but rather express very low levels of N-cadherin (Herrenknecht et al., 1991; Knudsen, K. A., personal communication, and see Fig. 8). HeLa cells display some epithelial characteristics, but only weakly; for example, they form contact-inhibited epithelial islands at low density and, as shown in Fig. 3, occasional adherens junctions, desmosomes, and tight junctions may be observed. Therefore, the machinery for the synthesis of these complex macromolecular structures ap-
pears undamaged and functional, but normally operates only at a low, sub-basal level that is not optimal for the creation and maintenance of a normal epithelium.

We observed that the expression of P₀ has the ability to profoundly affect the adhesive behavior and morphology of normally poorly self-adhesive HeLa cells. Relative to most other IgCAMS, the extracellular domain of P₀ is very small, consisting of a 124-amino acid segment that contains within it a single Ig-fold (Lai et al., 1987; Lemke et al., 1988). Homophilic interaction mediated by the mutual binding of this domain across the intermembrane cleft in peripheral nerve myelin yields a broad, continuous and absolutely regular 3–5-nm gap across the extracellular cleft that separates the Schwann cell bilayers (Raine, 1984). A similar size gap has been described by us for the P₀-expressing HeLa cells we used here (see D’Urso et al., 1990). From the morphological and aggregation data, we infer that a similar Ca⁺⁺-independent homophilic adhesive mechanism mediated by P₀ triggers dramatic ultrastructural changes in these transformed cells. Extensive membrane contact and cell–cell aggregation is accompanied by the restricted lateral localization of P₀, along zones of cell–cell contact (Fig. 2, Fig. 4, C and D).

The rapidity of P₀ expression cell aggregation under these conditions compares favorably with the time course of adhesive contact development in monolayers of keratinocytes or MDCK cells upon switching to high Ca⁺⁺ (1–2 mM), cell types in which preexisting components of junctional complexes are believed to assemble to cause epithelialization (Watt et al., 1984; Mattey and Garrod, 1986; Mattey et al., 1990). However, in cells such as keratinocytes, mature desmosomes are assembled within a very short time after cell–cell contact is established (Hennings and Holbrook, 1983; Watt et al., 1984) because all polypeptide components are apparently already present in sufficient quantity to yield the rapid assembly of desmosomes under the right conditions. Other studies have shown that the initial events in the process of reassembly of a previously formed epithelial monolayer do not require either the induction of relevant genes or de novo protein synthesis of junctional components (Pasdar and Nelson, 1988a,b; Hennings and Holbrook, 1983), although long term development relies heavily on new protein synthesis (Mattey et al., 1990). These studies used epithelial cells grown under culture conditions either of low Ca⁺⁺ or low cell density, in which preexisting intracellular pools of junctional components are present that can be readily mobilized and assembled once cell–cell adhesion has been initiated. In contrast, the generation of the epithelial phenotype in the HeLa cell line used in our studies fundamentally differs in that initial close membrane contact is brought about by the expression of a “foreign” homophilic adhesion molecule by these cells.

Our most striking observation was that by obligating HeLa cells to adhere to one another along extensive areas through the surface expression of P₀, the formation of certain junctional complexes characteristic of normal epithelial tissues was dramatically enhanced. P₀-P₀ interaction in this context clearly acts adhesively before, or in conjunction with, what has been traditionally viewed as the sine qua non for epithelialization; i.e., the cadherin-catenin adhesion system (Watabe et al., 1994). Our data reveal that newly formed linear arrays of desmosomes, whose major integral membrane components (desmocollin and desmoglein) are cadherins (Goodwin et al., 1990; Holton et al., 1990; Koch et al., 1990; Mechanic et al., 1991), arise progressively within an extensive, previously formed island of P₀ adhesion (Fig. 6), analogous to the “zippering” spread of stable contacts that have been observed between epithelial cells during early stages of cell–cell adhesion (McNeill et al., 1993). Since P₀ adhesion allows membranes to engage at a minimal distance (3–5 nm), and the mature desmosome separates bilayers by ~30 nm, it may be expected that P₀-mediated adhesion cannot continue to operate where mature desmosomes “embedded” in the P₀ adhesion zone are present.

In addition to desmosome formation, P₀ induced cell–cell adhesion also dramatically enhances the organization of tight junctional and adherens junction associated proteins at the lateral contact sites. Although there is clearly some low level expression of adhesion molecules in these cells (e.g., Fig. 7, A and C), normally their contribution to cell–cell adhesion in this carcinoma must be minimal. The expression of a strong, functional, adhesion molecule such as P₀ in HeLa cells therefore seems to convey the additional adhesivity necessary for the formation of junctions characteristic of epithelial tissues. Similarly, when the adhesive properties of S180 mouse sarcoma cells were enhanced by expression of L-CAM, a cadherin, or when these cells were agglutinated with lectins, functional gap junctions formation was increased (Mege et al., 1988), a result of the appropriate phosphorylation of connexin 43 (Musil et al., 1990). However, when the neural cell adhesion molecule (NCAM), a member of the immunoglobulin gene superfamily, was expressed in S180 cells, functional gap junctions did not develop, even though NCAM has previously been shown to have certain adhesive properties (Edelman et al., 1987; Williams and Barclay, 1988). “Strong” cell–cell adhesion, such as that brought about by cadherins (Shapiro et al., 1995) or the immunoglobulin P₀ may therefore be necessary for the marked effects on the cytoarchitecture and physiology of the cells involved. We should emphasize that we did not attempt to test for functionality of any of the junctional elements we examined.

The subcellular mechanisms by which P₀ brings about dramatic changes in HeLa cells have not been directly examined here, but it can be adduced from these studies, and from what is known about P₀ function in Schwann cells, that this molecule acts initially to bring adjacent plasma membranes and their integral membrane proteins into close apposition, allowing the formation of stabilizing junctional complexes and perhaps eliciting second messenger pathways. In this model, P₀ acts solely to overcome the “functional repulsion” that seems to exist between normal HeLa cells. Once initiated, cell–cell adhesion is undoubtedly reinforced by adhesion-based structures, the desmosomes and adherens junctions which use cadherins as major integral membrane adhesion molecules. Although N-cadherin (Figs. 8 and 9) is not usually recognized as a key contributor to epithelial cell–cell adhesion, it may play a critical role in the morphological changes seen in P₀-expressing HeLa cells. When PC12 cells, which normally express N-cadherin, are placed in contact with N-cadherin-expressing NIH3T3 cells, they differentiate, assuming a
neuronal morphology (Doherty et al., 1991). This transition can be mimicked by direct activation of second messenger systems, suggesting that the homophilic association of N-cadherin is one activator of an intracellular signaling pathway. The cytoplasmic domain of N-cadherin is known to bind to catenins (Ozawa and Kemler, 1992; Hirano et al., 1992). Cytoskeletal proteins that may be involved in the second messenger cascade that may be activated in these cells after Po initially brings adjacent plasma membranes into close apposition. Speculatively, no matter how the adhesion cascade is initially triggered, induction of an intracellular messenger system that is common to all epithelia, and that controls the implementation of the adhesion program may rapidly follow.

While recent results demonstrate that the cytoplasmic domain of Po is necessary for aggregation of Po-transfected cells in suspension (Wong and Filbin, 1994), suggestive of interactions between Po and the cytoskeleton, the cytoplasmic domain of Po has no known intracellular signaling properties. In Schwann cells, Po is absolutely and precisely targeted after synthesis to the forming compact myelin plasma membrane domain, effectively removing it from cell membrane circulation. Furthermore, the intact cytoplasmic segment of Po may be nonessential for triggering of the adhesion response in HeLa cells, since in preliminary experiments we have observed that an engineered Po extracellular domain that is missing the cytoplasmic segment but is anchored to the plasma membrane via a glycosylphosphatidylinositol moiety, can mediate similar changes in HeLa cell morphology. In summary, our results support a model in which Po, a strong membrane adhesion molecule, by maintaining close membrane contact, allows the development of stabilizing adhesive junctions and the interaction of molecules possibly involved in second messenger signaling. The dramatic reorganization of tight junction–associated proteins, desmosomal proteins, and adherens junction proteins leads these transformed cells to display a differentiated, classic epithelial phenotype. Perhaps most important, the regeneration of this phenotype is correlated, at least in this cell type, with the engagement of the cadherin Ca++-dependent adhesion system and the sub cortical components through which they are able to transduce changes in cell morphology. Po-mediated compaction of peripheral nerve myelin may similarly affect the expression or localization of native myelin proteins (Doyle and Colman, 1993) including the recently described cadherin/catenin system in myelinating Schwann cells (Fannon et al., 1995). In HeLa cells, Po clearly initiates an adhesion cascade, but the sequence of events that follow have not been clarified as yet. One model, alluded to above, is that the close membrane apposition “enforced” by Po stabilizes the interaction of extremely low levels of cell surface cadherins (N-cadherin, desmocollin, and desmoglein) that would otherwise be insufficient to trigger epithelioid development of the monolayer. In this model, an increase in junctional proteins at cell surfaces ensues and common intracellular pathways are used to yield epithelialization.

On the other hand, Po may possibly have as yet unrecognized intracellular messenger properties itself that directly activate common adhesive programs, bypassing the cadherin adhesion cascade. Po therefore may simultaneously compact cell membranes and trigger the subsequent steps leading to a general enhancement of the epithelial program. In this scenario, the upregulation of junctional markers we observed at the cell surface would then be an indirect consequence of Po activation.

The authors thank Eric Hubel and Lazar Fidler for preparation of the figures. This is manuscript number 183 from The Brookdale Center for Molecular Biology.

This work was supported by grants to D. R. Colman (NINDS NS 20147) and to P. Cowin (GM 47429) from the National Institutes of Health. D. D’Urso is an Alexander von Humboldt Fellow on leave from the Laboratory of Pathophysiology, Istituto Superiore di Sanita (Rome, Italy).

Received for publication 7 December 1994 and in revised form 20 June 1995.

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