Regulation of lung epithelial cell morphology by cAMP-dependent protein kinase type I isozenzyme

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A MAJOR AIM OF LUNG CANCER research is to understand the molecular basis underlying physiological differences between transformed and nontransformed cells. Because of their common heritage, the E10/E9 and C10/A5 sibling pairs of mouse lung epithelial cell lines are convenient for elucidating how neoplastic conversion modulates phenotype. The E10 and C10 lines were established from normal lung explants and had alveolar type II cell features such as lamellar bodies and surfactant apoprotein immunoreactivity at early passage (41). E9 and A5 are their spontaneous transformants, respectively, and were selected on the basis of their resistance to growth inhibition by dexamethasone (42). E10 and C10 display characteristics typical of immortalized but nontransformed cell lines. These flat cells are nontumorigenic, exhibit contact inhibition, anchorage-dependent growth, and gap junctional intercellular communication (GJIC), and respond to hormonal and cytokine signals (22). In contrast, the round E9 and A5 cells are tumorigenic, free from contact inhibition, and anchorage-independent, exhibit minimal GJIC, and are relatively unresponsive to environmental stimuli. Analogous to these behavioral differences, the molecular phenotype between these sibling pairs also differs (22). For example, E10 and C10 contain the wild-type Kras protooncogene, express the Cdkn2a, Apc, and Mcc tumor suppressor genes in contrast to their transformed counterparts, and have elevated levels of hormone receptors and signaling effector molecules.

The E10 line grows as single stellate cells until confluence is achieved, whereas E9 cells are round and grow in colonies. Roundness indicates reduced attachment to the surface, and most cells in culture tend to round up before division; this sphericity of E9 cells may reflect the fact that E9 cells grow faster than E10 cells (22) and may even facilitate proliferation. These morphological distinctions, flat vs. round and single cells vs. colonial growth, are mediated in part by actin and vinculin. Actin microfilaments are important in the genesis of cell shape, in regulating cell motility, and at cell-cell and cell-substrate interfaces (37). Vinculin directly interacts with actin to link the cytoskeleton to the extracellular milieu (35). Decreased focal adhesions between a cell and its substratum can lead to the loss of adhesiveness and greater cell motility that are properties of tumor cells. Vinculin may be a tumor suppressor because its overexpression in tumor cells decreases their tumorigenicity and metastatic potential (32).

One means of regulating cellular morphology is via protein kinase A (PKA) (38), an enzyme central to many signal transduction pathways (2). A striking difference in PKA exists between the sibling lung cell lines. cAMP directly activates PKA, resulting in the phosphorylation of endogenous substrates and subsequent changes in cell physiology. The type I holoenzyme [protein kinase isozyme (PKA I)] is composed of two catalytic (C) subunits plus two regulatory (RI) subunits, whereas the type II holoenzyme (PKA II) consists of two C subunits and two RII subunits; the R

A. Malkinson. Regulation of lung epithelial cell morphology by cAMP-dependent protein kinase type I isozenzyme. Am J Physiol Lung Cell Mol Physiol 280: L1282–L1289, 2001.—Cell shape is mediated in part by the actin cytoskeleton and the actin-binding protein vinculin. These proteins in turn are regulated by protein phosphorylation. We assessed the contribution of cAMP-dependent protein kinase A isozenzyme I (PKA I) to lung epithelial morphology using the E10/E9 sibling cell lines. PKA I concentration is high in flattened, nontumorigenic E10 cells but low in their round E9 transformants. PKA I activity was lowered in E10 cells by stable transfection with a dominant negative RI subunit and was raised in E9 cells by stable transfection with a wild-type Ca catalytic subunit construct. Reciprocal changes in morphology ensued. E10 cells became rounder and grew in colonies, their actin microfilaments were disrupted, and vinculin localization at cell-cell junctions was diminished. The converse occurred in E9 cells on elevating their PKA I content. Demonstration that PKA I is responsible for the dichotomy in these cellular behaviors suggests that manipulating PKA I concentrations in lung cancer would provide useful adjuvant therapy.

stable transfectants; actin; vinculin
subunit specifies isozyme type. There are two isoforms of each R subunit (RIα, RIβ, RIα, and RIββ) and two C subunit isoforms (Ca and Cβ) in mice. R subunits inhibit catalytic activity and participate in C subunit localization. cAMP binding to R subunits releases the C subunits, and the resultant conformational change exposes their catalytic site. Although lung cell lines contain similar amounts of PKA II, E10 and C10 have higher levels of PKA I protein and RIα mRNA than do their transformed counterparts (14, 29). The siblings also differ in the sensitivity of PKA subunit mRNA to proteins that regulate message stability (16). As a result of this PKA I deficit, phosphorylation of specific endogenous protein substrates was reduced when intracellular cAMP content was raised in intact E9 cells stimulated with forskolin and when cAMP was added to broken E9 cell extracts (15).

We hypothesize that the morphological differences between E10 and E9 reflect their differential PKA I contents, and we have tested this by reciprocally modifying PKA concentrations in these cells using a series of DNA constructs derived by McKnight and colleagues (4, 26, 45). After PKA I concentration was lowered in E10 stable transfecants and raised in E9 transfecants, we investigated the anatomic consequences. Dramatic reciprocal changes in cellular morphology and cell-cell adhesiveness were observed. E10 transfecants depleted of their PKA I resemble E9 more than their parental E10 cells; E9 clones with elevated PKA I levels are structurally more similar to E10 than to their parental E9 cells. The construction of these transfecants and their phenotypes, including their actin and vinculin distributions, are herein described. These transfecants constitute a resource for assessing the roles of PKA in lung cell physiology.

MATERIALS AND METHODS

Cell culture. The immortalized but nontumorigenic E10 cell line developed from a BALB/c mouse lung explant is alveolar type II cell derived (41). Tumorigenic E9 cells are spontaneous transformants of E10 and therefore very similar (42). The use and characterization of these cell lines were reviewed recently (22). Cells were grown in CMRL-1066 medium (Life Technologies, Gaithersburg, MD) that was supplemented with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA), penicillin G (10 μg/ml), and amphotericin (0.25 μg/ml) at 37°C in a humidified atmosphere of 5% CO2-95% air and trypsinized just before confluence for passaging. Plasmids were maintained in Geneticin (G418, 255 μg/ml). Cells were photographed using an Olympus CK inverted microscope fitted with an Olympus 35-mm camera and Kodak Tmax 100 black and white film.

Plasmids. PKA subunit-containing plasmids were kindly provided by Dr. G. Stanley McKnight (Dept. of Pharmacology, University of Washington, Seattle, WA). E10 cells were transfected with the plasmid MT-REVAB-neo (4) that contains a neomycin/G418 resistance gene expressed from an SV40 promoter, PUC13 sequences for maintenance in bacteria, and cDNA encoding a mutant RIα subunit. Mutations in each of the two cAMP-binding sites cause RIα to act as a dominant negative inhibitor of C-subunit activity (4); this REVAB (RAB) gene product associates with C subunit but cannot bind cAMP. RAB protein therefore releases C subunit with low efficiency, maintaining PKA holoenzyme in an inactive state. Expression of this gene is regulated by the metallothionein promoter and can be induced by growing cells in the presence of Zn2+. To induce expression of the transfected genes, cell lines were treated with 100 μM ZnSO4 in the above culture medium for at least 24 h. Transfectants exhibited maximal induction of transfected gene mRNA within 4 h after treatment with Zn2+, a level that remained constant for at least 24 h (data not shown).

E9 cells were transfected with the MT-CevEV-neo plasmid (45), which contains a neomycin/G418 resistance gene whose expression is controlled by an SV40 promoter, PUC13 sequences for maintenance in bacteria, and cDNA encoding wild-type Ca subunit. The CoEV (CEV) gene, which is also regulated by the metallothionein promoter, produces an mRNA that is shorter than that transcribed from the endogenous gene (1.7 kb rather than 2.4 kb), so it can be easily detected by Northern blot. The transcript is truncated at the 3′-end but retains all of the coding sequences (45). Overexpression of this Ca subunit leads to preferential accumulation of PKA I holoenzymes over PKA II (26).

Empty vector control lines were developed by transfecting E10 and E9 with pcDNA3 plasmid (Invitrogen, Carlsbad, CA) containing a neomycin/G418 resistance gene controlled by an SV40 promoter, PUC19 sequences for maintenance in bacteria (similar to PUC13), and additional sequences needed for mammalian expression of an inserted gene to construct cell lines that are G418 resistant but without PKA genes.

The transfected E10 cells are designated RAB#, E9 transfecants are CEV#, and the empty vector transfecants are E10PC# and E9PC#. For each transfected cell line, we isolated several empty vector control lines (E10PC# or E9PC#) and three lines that expressed the appropriate gene, RAB16, RAB20, or RAB31 and CEV1, CEV5, or CEV9. More than one transfected clone was analyzed per cell line to ensure that phenotypic differences are a function of the transfected gene and do not depend on where they are randomly inserted into the parental genome. The E10PC lines were identical to each other and used interchangeably as were the E9PC lines.

Transfections. Stable transfecants were made by introducing DNA into cells using calcium phosphate as described earlier (34). Cells were grown to 50% confluence and fed 3–4 h before DNA was added. Plasmid DNAs were linearized and added at three different concentrations (1, 5, and 10 μg) to ensure that at least one plate had an optimal colony density. Transfectants were selected with medium containing 450 μg/ml Geneticin. Untransfected cells died within 8–14 days from the start of this procedure, and surviving colonies were isolated and expanded. E9 cells were readily transfectable and produced well-isolated discrete colonies by 8 days. E10 cells did not produce colonies even though resistant individual cells could be seen on the plates. If the E10 transfecants were first treated with conditioned medium plus 450 μg/ml Geneticin, however, colonies were obtained. Conditioned medium was prepared by growing untransfected E10 cells to 80% confluence, removing and filtering the medium, and then mixing it 1:1 with fresh medium.

Northern blots. RNA was isolated from cells grown to 80–100% confluence using the RNeasy kit (QIAGEN, Valencia, CA) and quantified spectrophotometrically. Plasmid DNA (kindly provided by Dr. G. S. McKnight) was prepared using the Plasmid Maxi Kit (QIAGEN) and probes were prepared from the plasmids by excising the PKA genes. MT-CQR-neo, used to label Ca mRNA from the CEV clones, was treated with AapI and NcoI endonucleases to generate a restriction fragment containing Co. The MT-REV-neo plasmid, used to produce the probe for labeling RIα mRNA, was prepared from the plasmids by excising the PKA genes.
prepared from RAB clones, was treated with XbaI and PstI to generate a restriction fragment containing R1A. After endonuclease digestion, DNA fragments were separated by electrophoresis through a 1% agarose gel (FMC BioProducts, Chicago, IL), the band visualized with long-wavelength ultraviolet light was excised from the gel, and agarose was removed by digestion with β-agarase. DNA probes were resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) to a final concentration of 50 μg/ml.

Northern blots were performed as described (43). Ten micrograms of total RNA were loaded onto a 1% agarose-2.5% formaldehyde gel and electrophoresed. RNA was capillary-transferred to a nylon membrane in 10× SSC buffer (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0). DNA probes (50–100 μg) were labeled with [α-32P]cytidine 5’-triphosphate using the RadPrime labeling kit (Life Technologies) to a specific activity of 108 counts per minute (cpm) and hybridized with membranes overnight. After a wash, blots were exposed to X-ray film (Kodak X-Omat) or analyzed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**PKA activity assays.** Cells were grown to 80–100% confluence with and without 100 mM Zn2+ for 24 h before homogenization, washed in PBS, harvested by scraping, and resuspended in 50 μl of 15 mM Tris-HCl, pH 7.5, 2 mM EDTA, 20% glycerol, 100 μg/ml leupeptin, and 2 μg/ml aprotinin per plate. The cells were disrupted by sonication and centrifuged at 16,000 g for 30 min to separate particulate and cytosolic fractions, and protein concentrations were determined (18). Activity assays were performed using cytosolic fractions prepared from each of three plates per cell line as directed by either the Pierce colorimetric PKA assay kit (Rockford, IL) or the Promega Signa-TECT PKA assay system (Madison, WI) with similar results; each plate was assayed in triplicate. In the Pierce colorimetric kit, the PKA-specific substrate Kemptide is prelabeled with a colorimetric dye, whereas in the Signa-TECT kit, Kemptide is unlabeled. Cell fractions were incubated with Kemptide at 37°C for 30 min in the supplied reaction buffer containing ATP or [γ-32P]ATP in the Signa-TECT kit. The reaction mixtures were then applied to anionic supports. Only phosphorylated peptide binds to this support and can be eluted to yield either colorimetrically or radioactively labeled phosphorylated Kemptide, which is then measured using a fluorometric plate reader or scintillation counter. Significance of the difference between PKA activity of the vector control cells (in the absence of Zn2+) compared with the transfected cells was determined by Student-Newman-Keuls analysis. The Pierce colorimetric assay was used to screen transfecants, and results were confirmed using the Signa-TECT kit.

**Immunocytochemistry.** Cells were cultured on four-chambered slides (Falcon, Franklin Lakes, NJ). For each cell line, two chambers contained untreated cells and two chambers contained 100 mM Zn2+-treated cells and were processed as described (6) with the following modifications. Cells were fixed initially in 3.7% formaldehyde dissolved in PBS (in the absence of Mg2+ or Ca2+ salt) with 0.1% Tween 20 for 20 min; 0.25% Triton X-100 was added for 5 min to permeabilize the plasma membranes. For actin staining, cells were incubated overnight at 4°C with phalloidin labeled with Oregon Green 514 (Molecular Probes). For vinculin staining, cells were incubated overnight at 4°C with a mouse monoclonal anti-vinculin antibody (Sigma, St. Louis, MO) and treated with goat anti-mouse secondary antibody labeled with Alexa 594 (Molecular Probes, Leiden, The Netherlands) for 2 h at 37°C. Immunostaining was visualized with a ×40 objective using a Nikon Microphot photomicroscope equipped with epifluorescent optics. Photographs were taken using Kodak 400 ASA color slide film.

**RESULTS**

**E10 and E9 transfectants express the PKA cDNA constructs.** RNA was isolated from control and transfected cell lines and analyzed by Northern blot (Fig. 1). Data from the E10PC31 and E9PC14 control lines were identical to that from their E10 and E9 parents. Although the RAB plasmid did not produce an mRNA that was qualitatively distinguishable from the endogenous 3.2-, 3.0-, and 1.8-kb RIα bands, the transfectants expressed more of the smallest size band (Fig. IA). RAB16, RAB20, and RAB31 all produce more 1.8-kb message than the control, and this band increased an additional threefold or greater over E10PC31 levels when cells were treated with Zn2+. The plasmid construct may only be capable of producing the smaller message. It is not surprising that the RAB clones produced elevated levels of this band even in the absence of Zn2+ exogenously added to the medium because the metallothionein promoter exhibited weak constitutive activity.

Figure 1B shows the Northern analysis for the E9PC6 empty vector control and for the CEV1, CEV5, and CEV9 lines. The CEV plasmid produced an mRNA of 1.7 kb, which was easily distinguishable from the endogenous Ca mRNA of 2.4 kb. Clearly, all three CEV clones produced the CEV transcript even in the absence of Zn2+. CEV mRNA content was elevated when the cells are grown in the presence of Zn2+.

**PKA activity in RAB and CEV transfectants.** The result of reciprocally altering PKA mRNA contents in these cell lines should be manifested at the level of enzyme activity, the most accurate assessment of PKA function. Both RAB16 and RAB31 had decreased PKA activities, and these were further reduced to only 25–30% of control levels when the cells were grown in the presence of Zn2+. The RAB20 line showed a more modest decrease in PKA activity in the absence of Zn2+, with a slight additional decrease on Zn2+ induction (Fig. 2A). Such phenotypic variation is normal among independently isolated stable transfectants. CEV1 cells exhibited similar PKA activity to E9PC14 in the absence of Zn2+, but this increased to a level that was fourfold higher in Zn2+-treated cells (Fig. 2B). CEV5 displayed more PKA activity than the void vector in the absence of Zn2+ and a further fourfold increase above control in the presence of Zn2+. CEV9 PKA activity was substantially higher than that in the other two clones, with 10-fold more PKA activity in the absence of Zn2+ and 30-fold more in the presence of Zn2+.

**Effect of altered PKA on cell shape and colony morphology.** Figure 3 illustrates the morphology of cells grown in the absence (A–D) or the presence (E–H) of Zn2+ by light microscopy. The parental E10 and E9 lines were indistinguishable from the empty vector control lines, E10PC31 and E9PC14, respectively (data not shown). E10PC31 grows predominantly as single stellate cells until they become confluent (Fig. 3, A and
In contrast, the RAB31 clone retains the stellate shape but grows in colonies (Fig. 3, B and F), which is reminiscent of the growth pattern of E9 cells. These cellular aggregates become larger when cells are grown in the presence of Zn$^{2+}$. All three RAB clones displayed this altered growth pattern, RAB16 cells to the least extent.

E9PC14 exhibits a typical E9 morphology, i.e., round cells that grow in tight clumps in which single cells are rarely present (Fig. 3, C and G). The morphology of CEV5 is more typical of E10 (Fig. 3, D and H) in that the cells are stellate rather than round and tend to pull away from each other rather than remaining tightly associated. All CEV lines showed a similar morphology change.

Decreasing PKA I content in nontransformed E10 cells caused them to take on the morphological and cell-cell adhesive properties of E9, whereas increasing the PKA I concentrations in E9 cells compelled them to behave more like E10. The reciprocal nature of these changes emphasizes that the intracellular concentration of PKA I affects cell-cell associations.
Effect of altered PKA I expression on actin fiber distribution.

To explore the basis of these phenotypic changes, the transfectants were stained for filamentous (F) actin (6). E10 cells exhibit elongated stress fibers that form parallel arrays across cells, whereas E9 has few detectable filaments and has diffusely distributed actin (22). Figure 4 shows epifluorescence photographs of cells stained for F-actin that were grown in the absence (A–D) or presence (E–H) of Zn\(^{2+}\). PC control cells were identical to the parental E10 and E9 lines (Fig. 4, A and E). In E10PC31 cells, actin is in elongated bundles aligned across more than one cell as previously observed with E10 (6). The RAB transfectants, however, manifest fewer fibers and a diffuse staining pattern (Fig. 4, B and F) that is characteristic of transformed E9 cells. All RAB clones displayed actin disorganization.

The actin distribution in E9PC6, the empty vector control, was E9-like: diffuse staining, little fiber formation, and a punctate appearance at cell junctions (Fig. 4, C and G). In contrast, CEV5 had stress fiber bundles not seen in control E9 lines (Fig. 4, D and H). All CEV lines exhibited actin fiber formation, and some clones demonstrated additional stress fiber formation in the presence of Zn\(^{2+}\) (Fig. 4, D and H; CEV1 data not shown). CEV9 did not exhibit stress fibers in the presence of Zn\(^{2+}\). CEV9 was so sensitive to Zn\(^{2+}\) that viability was difficult to maintain, probably because of its excessive kinase activity (Fig. 2D).

Decreasing PKA I content in E10 cells diminished actin stress fiber formation, creating a more diffuse actin staining pattern that is similar to that of E9 cells. Increasing the concentration of PKA I in E9 cells enhanced actin stress fiber formation, and these transfected cells resembled E10.

Effect of altered PKA expression on vinculin immunostaining.

To further characterize the morphological changes, we examined vinculin staining patterns in the

Fig. 4. Actin staining of E10 cells transfected with mutant RIα and E9 cells transfected with wild-type Ca as shown by epifluorescence microscopy of cell lines not treated (+Zn) or treated for at least 24 h with ZnSO\(_4\) (+Zn). A: E10PC31 -Zn. E: E10PC31 +Zn. B: RAB31 -Zn. F: RAB31 +Zn. C: E9PC14 -Zn. G: E9PC14 +Zn. D: CEV5 -Zn. H: CEV5 +Zn.
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Fig. 5. Vinculin staining of E10 cells transfected with mutant RI and E9 cells transfected with wild-type Co as shown by epifluorescence microscopy of cell lines not treated (-Zn) or treated for at least 24 h with ZnSO4 (+Zn). Cells were first incubated with a mouse monoclonal antibody that recognizes vinculin and then stained with a goat anti-mouse secondary antibody conjugated to Alexa 594 to visualize vinculin. A: E10PC31 -Zn. E: E10PC31 +Zn. B: RAB31 -Zn. F: RAB31 +Zn. C: E9PC14 -Zn. G: E9PC14 +Zn. D: CEV1 -Zn. H: CEV1 +Zn.

transfectants. Vinculin interacts with actin at focal adhesion plaques to regulate cell-to-substrate attachment and motility (38). E10 cells show a distinct pattern of vinculin "bars" at the edges of the cells (6). E9 had virtually no vinculin bars, demonstrating instead a faint diffuse staining pattern with occasional punctate staining at the cell periphery (data not shown). Figure 5 shows epifluorescence photographs of cells stained for vinculin after growth in the absence (A–D) or presence (E–H) of Zn2+.

The vinculin staining pattern of E10PC31 was indistinguishable from that of the parental E10 line (Fig. 5, A and E), with heavy staining of vinculin bars at the cellular periphery. Most cells in the field of view contained vinculin bars. RAB clones exhibited a modest difference in vinculin staining compared with their parental E10 cells (e.g., RAB31, Fig. 5, B and F), consisting of fewer but more elongated bars than in control cell lines. The E9PC14 control line displayed no vinculin bars (Fig. 5, C and G) as is typical of the staining pattern of E9 (data not shown). The CEV cells had bars at the cell periphery that apparently increased in number in the presence of Zn2+ (Fig. 5, D and H). CEV9 cells formed vinculin bars in the absence of Zn2+ but grew poorly in the presence of Zn2+, making it difficult to observe vinculin staining (data not shown). Whether these differences in staining were due to differences in actual cellular vinculin content or to a difference in vinculin organization was not tested.

Decreasing the PKA I content of E10 modestly decreased the number of vinculin bars, which is a trend toward the vinculin staining pattern of E9 cells. Conversely, increasing PKA I concentrations in E9 gave rise to distinct vinculin bars similar to those found in E10.

DISCUSSION

To test the hypothesis that PKA regulates the distinct morphologies and growth patterns of E10 and E9 cells, we stably transfected E10 cells with a dominant negative RIα construct to lower their PKA I content and raised PKA I concentrations in E9 cells with wild-type Co cDNA. These PKA constructs were previously used to demonstrate cAMP modulation of pulmonary cystic fibrosis transmembrane conductance regulator (CFTR) activity via PKA-mediated CFTR phosphorylation and stimulation of CFTR gene expression (25) and as evidence for the causal involvement of PKA in lung metastasis (46). The amount of the smallest RIα mRNA species was elevated in the three G418-resistant E10 clones examined following Zn2+ induction (Fig. 1), and this resulted in lower PKA activity (Fig. 2). The transfectants became rounder and clustered together (Fig. 3), in contrast to their E10 parental cells, which normally grow as single stellate cells. The number of microfilaments decreased as cellular actin became disorganized (Fig. 4), and the vinculin bars typically present at the cellular periphery were lost (Fig. 5). E9 was transfected with wild-type Co subunit to elevate PKA I content. Truncation of the 3'-end of the Co cDNA construct allowed detection of a mRNA of altered size, whereas the translated regions and hence the biological activity is retained. This is the reason mRNA rather than PKA protein concentrations were examined to demonstrate that transfection had occurred. The smaller Co mRNA is present in the E9 transfectants, and its transcription is induced when cells are briefly exposed to Zn2+ (Fig. 1) as was PKA activity (Fig. 2). Addition of exogenous Zn2+ caused these transfectants to adopt a more stellate morphology than the parental E9 cells, and these transfectants frequently grew as individual cells rather than in clumps (Fig. 3). This shape change correlated with increased actin microfilament formation (Fig. 4) and was accompanied by an apparent increase in the number of vinculin bars in focal adhesions at the plasma membrane (Fig. 5). The reciprocal nature of these changes in cellular structure and cell-cell interactions strongly implies that the engineered changes in
PKA I content are responsible for them. Both actin (37) and vinculin (44) are PKA substrates, and cAMP can enhance expression of actin genes (36).

This effect of PKA on the structure of E10 cells is the opposite of what happens when protein kinase C (PKC) is activated in E10 cells by phorbol ester treatment. With phorbol esters, E10 cells become rounder and ruffled within a few minutes (5). When activated PKC is degraded in a process initiated by calpain-catalyzed limited proteolysis, the original flat E10 cell shape is restored. These morphological changes are accompanied by altered actin and vinculin distributions similar to those observed herein when PKA I content is lowered (6). E9 cells are unresponsive to phorbol ester-induced shape changes (Dwyer-Nield LD, Dinsdale D, and Malkinson AM, unpublished results), probably due to their negligible PKC-α content (5), but the round PCC4 lung tumor-derived cell line flattened on phorbol ester exposure (29). Therefore, activating PKC in these nontumorigenic and tumor-derived cell lines reciprocally affected their morphology. The actions of PKA I and PKC on E10 cell structure are thus antagonistic. Multivalent protein kinase scaffolding proteins bind PKC and PKA into a multienzyme complex that regulates cytoskeletal structure (28). Delineating those protein substrates that are phosphorylated by each of these serine/threonine protein kinases and are responsible for these architectural modifications would be illuminating.

The relative amounts of PKA I and PKA II are altered when 3T3 fibroblasts are virally transformed (8). This observation, together with the rise in cAMP levels when mesenchymal cells reach confluence (39) and the reduced cAMP concentration on viral transformation (40), implies that high cAMP is associated with proliferative quiescence. Elevating cAMP content in glioma cells evoked differentiated characteristics, including anchorage dependence and contact inhibition of growth, a process called “reverse transformation” (31). Addition of dibutyryl (DB) cAMP to E10 cells inhibited their growth and increased the extent of GJJC (1). DBcAMP caused similar but greatly attenuated changes in E9 cells, probably because of their decreased PKA I content. Responses to the ubiquitous cAMP second messenger are cell-type specific (33). Just as cells differ in their growth response to cAMP, they also vary in how cAMP affects their morphology. In CHO cells, cAMP promotes actin polymerization into stress bundles (17), but cAMP disassembles actin filaments in African green monkey kidney cells (44). PKA may exert such bidirectional effects by shifting a cell from one Ras signaling module to another, e.g., from the proliferative extracellular signal-regulated protein kinase pathway to the p38 pathway, which is more closely associated with differentiation (27). Mutation of Kras initiates mouse lung tumorigenesis (11); E9 cells contain only mutant Kras, whereas E10 cells are wild type (30). This mutation may affect PKA expression, and, in turn, Ras signaling is modified by PKA (9). PKA expression changes during mouse lung ontogeny (23), and lung tumor cells assume the fetal rather than the adult PKA phenotype (21).

Reverse transformation is an attractive candidate for inclusion in novel cancer prevention and therapeutic strategies (3). It is therefore important to study a cell type whose neoplastic conversion leads to a clinically relevant cancer, to describe how cAMP influences its physiology under normal and neoplastic conditions, and to deduce the molecular mechanisms directing that response. More deaths are attributable to lung cancer than to the next most common solid tumors, colorectal, prostate, breast, and pancreatic, combined (13). Adenocarcinoma (AC) is the most frequent form of lung cancer in smokers and the only subtype that nonsmokers develop. The incidence of AC is increasing alarmingly (7), and AC patients seldom survive long after diagnosis. AC is a peripheral cancer of the lung largely derived from nonciliated bronchial Clara cells and alveolar type II cells (10). Mice develop lung tumors similar to human AC in their histology and molecular characteristics (10, 19, 20). Mutation of KRAS is the hallmark of one-third to one-half of human AC and most mouse lung tumors (20). Ras proteins alter cytoskeletal structure, gene expression, and cell-cell interactions (24). The ability of PKA to interfere with Ras signaling by phosphorylating Raf and thus blocking its stimulation by Ras (12) suggests a downstream mechanism for the observations herein presented. Because PKA content is an important determinant of lung epithelial cell shape and the extent of intercellular contacts, drugs that specifically modulate PKA expression may serve as adjuvants to traditional therapies for treating AC.

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