Regulation of Cytoskeleton Organization and Paxillin Dephosphorylation by cAMP

STUDIES ON MURINE Y1 ADRENAL CELLS

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Cyclic AMP induces corticosteroid production, differential gene transcription, and cell cycle arrest in adrenal cortex-derived Y1 cells. These responses follow a cAMP-controlled transformation in Y1 cell morphology: the conversion of flat epithelial cells into rounded, highly refractile cells with short processes. Little is known about effector proteins and mechanisms that link activated protein kinase A to the alteration in cell shape. We now report that cAMP causes rapid (1 min) and selective tyrosine dephosphorylation of paxillin, a focal adhesion protein. Paxillin is maximally dephosphorylated before other physiological effects of cAMP are detected in Y1 cells. Dephosphopaxillin translocates from focal adhesions to the cytoplasm as stress fibers vanish and F-actin accumulates in membrane ruffles and cytoplasmic aggregates. Remnants of focal adhesion complexes disassociate from the cell cortex and coalesce into large structures that contain aggregated F-actin. Pervanadate, an inhibitor of protein-tyrosine phosphatases, abrogates all effects of cAMP. Conversely, genistein-sensitive protein-tyrosine kinase activity is essential for establishing epithelial morphology and reversing effects of cAMP in Y1 cells. Thus, cAMP/protein kinase A (PKA) actions are initially targeted to focal adhesions and cortical actin cytoskeleton; paxillin is an early and unexpected downstream target in a PKA-mediated signaling pathway, and protein-tyrosine phosphatase activity provides an essential link between PKA activation and the control of cell shape.

The second messenger cAMP and its effector, protein kinase A (PKA), mediate the regulation of many processes including glycolysis, ion transport, gene transcription, and cell proliferation and differentiation (1–4). Increased intracellular levels of cAMP also cause a reorganization of the actin cytoskeleton and enriched at sites along lengthy microfilaments (stress fibers) or concentrated in the F-actin-enriched cell cortex, which is contiguous with the inner surface of the plasma membrane. To begin an analysis of these topics we addressed several basic questions: Is the cortical actin cytoskeleton a proximal site of cAMP/PKA action? If so, are focal adhesion protein complexes (which anchor actin filaments in the cell cortex) early downstream targets in the cAMP signaling pathway? Are the effects of cAMP on cell shape solely the result of PKA-catalyzed Ser/Thr phosphorylation of effector proteins, or are other signaling pathways involved?

We demonstrate that cAMP causes the rapid tyrosine dephosphorylation of the focal adhesion protein paxillin in Y1 cells. Subsequently, paxillin translocates to the cytoplasm, stress fibers vanish, and F-actin accumulates in cytoplasmic aggregates and membrane ruffles. Remnants of multiple focal adhesions coalesce into large complexes that include aggregated F-actin. Pervanadate inhibits these processes, whereas genistein prevents their reversal. Thus, protein-tyrosine phosphatase activity couples activated PKA to regulation of actin cytoskeleton organization in Y1 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine Y1 cells were cloned from an explanted adrenal cortex tumor (6). Y1 cells were grown in Ham’s F-10 medium containing 12.5% fetal bovine serum in an atmosphere of 7.5% CO2, 92.5% air (37°C). Fed, firmly adherent, 50–75% confluent Y1 cells were used as a standardized system for investigating the effects of cAMP on...
the organization of a pre-existing actin cytoskeleton and its associated focal adhesion complexes.

Preparation of Cell Extracts and Western Immunoblot Analysis—Y1 cells were washed twice with 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Next, cells were lysed at 0 °C by the addition of 0.5 ml of PEM buffer (35 mM PIPES, pH 7.1, 5 mM EGTA, 5 mM MgCl₂, 0.2 mM sucrose, 0.5% (v/v) Triton X-100, 2 mM Na₃VO₄, 0.1 mM NaF, 0.5 μg/ml aprotinin, and 5 μg/ml pepstatin) per 10-cm culture dish. After scraping, lysates were vortexed for 15 s and incubated on ice for 5 min. Samples of total lysate protein (0.1 mg) were mixed with SDS loading buffer (12) and heated for 10 min at 95 °C. Proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis (9% gel) and transferred to an Immobilon P membrane (Millipore Corp.) as described previously (12). Membranes were probed with anti-phosphotyrosine (anti-PY) IgG (1:1000, Transduction Laboratories) or anti-paxillin IgG (1:10,000, Transduction Laboratories) as described previously (13). Antigen-antibody complexes were visualized on x-ray film by enhanced chemiluminescence methodology (Amersham Corp.) (13).

Immunoprecipitation Analysis—Cell lysates were centrifuged at 12,000 × g for 10 min, and supernatant fractions were collected. Solubilized proteins (0.4 mg) were incubated with either 1.5 μg of anti-paxillin IgG or 3 μg of anti-PY IgG that was immobilized on protein A-Sepharose 4B beads (Zymed). After 1 h at 0 °C beads were harvested by centrifugation at 7,000 × g for 2 min. Protein A-Sepharose containing IgG-antigen complexes was washed five times with 0.3 ml of 50 mM PEM buffer, pH 7.4, containing 0.15 M NaCl, 2 mM Na₃VO₄, 50 mM NaF, 1 mM dithiothreitol and 1% Triton X-100. Proteins were released from the beads by the addition of 60 μl of SDS loading buffer and incubation at 95 °C for 10 min. Immunoprecipitated and nonprecipitated (supernatant) proteins were fractionated by denaturing electrophoresis and transferred to an Immobilon P membrane. Duplicate blots were probed with anti-paxillin or anti-PY IgGs and developed as indicated above.

Immunofluorescence Analysis—Y1 cells were grown to ~60% confluence on glass coverslips. Cells were fixed (4% paraformaldehyde) permeabilized (0.2% Triton X-100) and blocked (3% albumin) in PBS. Next, cells were incubated sequentially with anti-PY IgG (1:50 dilution), and 10 μg/ml fluorescein isothiocyanate (FITC) conjugated goat IgG directed against mouse immunoglobulins. Cells were washed with PBS/0.2% Triton X-100 between incubations. Cells were co-stained for F-actin by using 0.6 μg rhodamine-conjugated phallidin (Sigma). A second batch of Y1 cells was incubated with anti-paxillin IgG (1:500) and FITC-conjugated secondary antibodies and then co-stained with rhodamine phallidin. Y1 cells were co-stained for paxillin and PY-containing proteins by using the primary monoclonal antibodies described above in combination with either rhodamine-conjugated secondary antibodies directed against mouse IgG (the anti-paxillin IgG isotype) or FITC-tagged secondary antibodies that bind mouse IgGsp (the anti-PY IgG isotype). Fluorescence signals corresponding to anti-antibody or F-actin-phallidin complexes were collected with a Bio-Rad MRC 600 laser scanning confocal microscope as described previously (14).

Materials—Genistein and tyrphostin 25A were purchased from LC Laboratories. Phenylarsine oxide, albumin, Na₃VO₄, forskolin, 8-(4-chlorophenylthio)-cAMP, and ACTH were obtained from Sigma. Pervanadate was generated as described by Evans et al. (15).

RESULTS
Y1 epithelial cells firmly adhere to uncoated plastic dishes or glass coverslips (Fig. 1A, a). Treatment with 0.2 mM 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), a cell-permeable analog of cAMP, alters cell morphology. Within 3 min some cells begin to retract their membranes and assume a “rounder” shape. After 6 min, ~10% of the cells display a highly refractile central region that gives rise to short, incompletely retracted processes (Fig. 1A, b). Approximately 50% of the cells exhibit this shape after 10 min, and virtually the entire population completes the morphological transition by 30 min (Fig. 1A, c). Incubation with 20 mM ACTH or 20 μM forskolin, an adenylate cyclase activator, yields the same results. The speed with which Y1 cells alter their cytoskeleton suggests that post-translational modifications of proteins mediate the response to cAMP.

Proteins with apparent molecular weights of 130,000 and 76,000 account for most of the PY residues in Y1 cells (Fig. 1B, lane 1). Treatment of Y1 cells with ACTH, forskolin or CPT-cAMP resulted in dephosphorylation of the 76-kDa polypeptide within 1 min (Fig. 1B, lanes 3, 4, 7, 8, 11, and 12). The 130-kDa protein was not markedly dephosphorylated in ACTH- and forskolin-treated cells. Dephosphorylation of the 76-kDa protein precedes the cell shape transition and identifies this protein as an early downstream target of cAMP/PKA action.

The size and PY content of the 76-kDa protein suggested that it might be paxillin, a focal adhesion phosphoprotein (16, 17). Anti-paxillin IgG quantitatively precipitated a broad band of proteins by using the primary monoclonal antibodies described under “Experimental Procedures.” Incubation with anti-PY IgG that was immobilized on protein A-Sepharose 4B beads (Zymed). Flows for the indicated time periods. Subsequently, cells were lysed in a buffer containing 0.5% Triton X-100. Proteins from cell lysates were fractionated by denaturing electrophoresis and transferred to an Immobilon P membrane as described under “Experimental Procedures.” The membrane was probed with anti-PY IgG, and antigen-antibody complexes were visualized on x-ray film by chemiluminescence as described under “Experimental Procedures.”
cAMP Regulates Paxillin Dephosphorylation and Cell Shape

FIG. 2. Cyclic AMP stimulates the dephosphorylation of tyrosine residues in the focal adhesion protein paxillin. Control Y1 cells and cells incubated for 10 min with either 20 μM forskolin or 0.1 mM CPT-cAMP were solubilized with PEMS lysis buffer. Samples of solubilized proteins were incubated with either anti-paxillin IgG (lanes 2–7) or anti-PY IgG (lanes 8–13) that was immobilized on protein A-Sepharose 4B beads (see “Experimental Procedures”). Proteins precipitated with the beads (P), and polypeptides remaining in the supernatant solution (S) were size-fractionated by denaturing electrophoresis (9% gel) and transferred to an Immobilon P (IP) membrane. Lanes 1 and 14 received total proteins (T) from control and CPT-cAMP-treated Y1 cells, respectively. Replicate Western blots were probed with anti-paxillin (α-Pax) (A) and anti-PY (α-PY) IgGs. Immunocomplexes were visualized by enhanced chemiluminescence methodology. Only the relevant portion of each immunoblot is shown. Phospho Pax, phosphopaxillin.

period. However, treatment with genistein for 8 h produced a rounded phenotype in 20% of Y1 cells; after 14 h of incubation with genistein nearly 90% of Y1 cells resembled cells treated with CPT-cAMP for 30 min (Fig. 1A, c). The PY content of paxillin was slightly diminished at 8 h (not shown), whereas the protein was predominantly dephosphorylated after incubation with genistein for 14 h (Fig. 3A, lanes 4, 7, and 8). These data suggest that PY on paxillin is hydrolyzed very slowly in unstimulated cells. Thus, a paxillin tyrosine kinase is not a likely target for rapid PKA-mediated signaling.

Paxillin is phosphorylated (Fig. 3A, lane 3), and Y1 cells revert to their epithelial morphology (Fig. 3B) 20 min after the removal of CPT-cAMP. However, genistein blocked both processes (Fig. 3A, lanes 4–6; cell shape identical to that shown in Fig. 3B, a). Thus, phosphorylation of paxillin may be a requisite step in the re-establishment of the epithelial phenotype of Y1 cells.

Pervanadate, which potently inhibits PTPs (19, 20), suppressed cAMP-stimulated paxillin dephosphorylation in short (Fig. 3C) and long term (tested up to 1 h) experiments. Cells did not alter their shape (Fig. 4 g–h), and paxillin became a sharp (maximally phosphorylated) band on Western immunoblots (Fig. 3C, lanes 3 and 4). Although pervanadate may partially activate certain tyrosine kinases (19, 20), results obtained with genistein (see above) suggest that increased tyrosine phosphorylation is not required for the cAMP-controlled morphological transition. Moreover, phenylarsene oxide, a PTP inhibitor that does not activate tyrosine kinases, also blocked effects of cAMP in short term experiments.

Y1 cells contain actin stress fibers (Fig. 4, upper panel, a’ and b’) and focal adhesions that stain with antibodies directed against either PY or paxillin (Fig. 4, upper panel, a and b). CPT-cAMP promotes the rapid translocation of a substantial proportion of paxillin from focal adhesions to the cytoplasm (Fig. 4, d and p). As paxillin redistributes, stress fibers become disorganized and F-actin is concentrated in membrane ruffles and intracellular aggregates (Fig. 4, c’ and d’), although remnants of focal adhesions are evident (Fig. 4c). After 30 min most of the paxillin is diffusely dispersed in the cytoplasm (Fig. 4f).

FIG. 3. PTP activity is required for the cAMP-regulated rearrangement of the cytoskeleton, whereas tyrosine protein kinase is essential for the restoration of epithelial morphology and the basal state of paxillin phosphorylation. A, Y1 cells were incubated with or without 0.2 mM CPT-cAMP for 10 min. Replicate plates of CPT-cAMP-treated cells were either processed immediately after exposure to the cAMP analog or incubated for an additional 20 min in medium lacking CPT-cAMP. Cell proteins (0.1 mg) were size-fractionated in an SDS-polyacrylamide gel (9%) and transferred to an Immobilon P membrane (see “Experimental Procedures”). The blot was probed with anti-PY IgG. Lanes 1 and 4 received proteins from Y1 cells that were not exposed to CPT-cAMP; lanes 2 and 5 contained proteins from cells harvested immediately after exposure to CPT-cAMP; and lanes 3 and 6 received proteins from cells that were incubated in the absence of CPT-cAMP for 20 min after a 10-min exposure to the cAMP analog. Proteins analyzed in lanes 4–6 were obtained from cells that were maintained in the presence of 0.3 mM genistein (60 min of preincubation plus experimental manipulation in the presence of the drug). Proteins in lanes 7 and 8 were derived from Y1 cells that were chronically maintained in medium supplemented with 0.3 mM genistein for 7 and 14 h, respectively. B, Y1 cells were preincubated with CPT-cAMP for 30 min to generate refractile cells with neurite-like processes. The morphology of a typical cell was monitored by real time video microscopy after feeding the cells with fresh medium lacking CPT-cAMP ana
glog. Frames obtained at 0, 6, 10, and 20 min (a–d, respectively) after removing CPT-cAMP document the rapid restoration of epithelial morphology. C, Y1 cells were preincubated 10 min with or without 0.2 mM pervanadate. Cells were then incubated in the presence or the absence of 20 μM forskolin for 10 min as indicated. Protein samples were prepared and analyzed as described under “Experimental Procedures.” The blot was probed with anti-paxillin IgG.

DISCUSSION

Shape and functions of Y1 adrenal cells are controlled by hormones that activate PKA (6–10). When the cAMP concentration is low, Y1 cells have a flat epithelial shape that receives structural support from a network of actin filaments (Fig. 4, upper panel, a’ and b’). At the cell cortex, the filaments are anchored by multi-protein complexes known as focal adhesions (Fig. 4, upper panel, a and b) (21, 22). Focal adhesions mediate...
cAMP Regulates Paxillin Dephosphorylation and Cell Shape

Fig. 4. Cyclic AMP induces the translocation of paxillin, aggregation of PY-containing proteins, and rearrangement of the actin cytoskeleton in Y1 cells. Upper panel, Y1 cells were grown on coverslips and treated with 0.2 mM CPT-cAMP for 0 (a, a', b, b', g, and g'), 10 (c, c', d, d', h, and h'), or 30 min (e, e', f, and f'). Cells shown in g, g', h, and h' were preincubated with 0.2 mM pervanadate for 10 min, and the same concentration of pervanadate was used during a subsequent 10-min experimental incubation with CPT-cAMP. Cells were then fixed, permeabilized, and blocked with 3% albumin. Y1 cells in a, c, and e were incubated sequentially with anti-PY IgG and 10 μg/ml FITC-conjugated, goat IgGs directed against mouse immunoglobulins. The same cells (a', c', and e') were co-stained for F-actin by using rhodamine-conjugated phalloidin. Y1 cells in b, d, f, g, and h were incubated with anti-paxillin and FITC-conjugated secondary antibodies and then co-stained with rhodamine-tagged phalloidin (b', d', f', g' and h'). Fluorescence signals corresponding to antigen-antibody (green) or F-actin-phalloidin (red) complexes were collected with a Bio-Rad MRC 600 laser scanning confocal microscope (see “Experimental Procedures”). Bar, 10 μm. Lower panel, Y1 cells were co-stained for proteins containing PY (green) and paxillin (red) after incubation with 0.2 mM CPT-cAMP for 10 (a and a') or 30 min (b and b').

The dephosphorylation reaction precedes depletion of paxillin from focal adhesions, disassembly of adhesion plaques, disorganization of stress fibers, and rearrangement of the actin cytoskeleton. Thus, cAMP/PKA actions appear to be initially targeted to the actin cytoskeleton and focal adhesions in the cell cortex. Paxillin is a previously unappreciated, early downstream target in a PKA-mediated signaling pathway.

Acute regulation of paxillin phosphorylation is not exerted at the level of tyrosine kinase(s). Rather, cAMP elicits an apparent increase in PTP activity. Perhaps PTPs directly phosphatylates and activates a PTP. Ser59 of PTP-PEST is phosphorylated by PTK in vitro and in cells (24). However, this modification reduced PTP-PEST activity by ~30% in cell extracts. Although the exact regulatory significance of serine phosphorylation in PTP-PEST and other PTPs remains to be determined, the data indicate that PTPs can be direct targets for PTK in intact cells. Alternatively, PTK may phosphorylate paxillin within the consensus substrate sequence RGRSL (25). If PTK-catalyzed phosphorylation alters the conformation of paxillin, its accessibility to regulated or constitutively active PTPs may be increased. More complex mechanisms involving phosphorylation of activators or inhibitors of PTPs are also possible (26).

Although exact mechanisms remain to be determined, it is evident that cAMP (PKA) regulates disassembly of focal adhesions and dissolution of actin filaments as well as the PY content of paxillin. Effects of cAMP on Y1 cells are blocked by PTK inhibitors. Moreover, tyrosine protein kinase activity is essential for reassembly of F-actin-focal adhesion complexes, regeneration and recruitment of phosphopaxillin to the cell cortex, and restoration of epithelial shape of Y1 cells when cAMP levels decline. Because injection of recombinant PTPs into Y1 cells produces the phenotype shown in Figs. 1A (c) and 4 (c–f), it appears that PTK activation and/or translocation is a necessary and sufficient step in the regulation of actin cytoskeleton organization and cell shape. Coordinated control of paxillin dephosphorylation and cell morphology via PKA-mediated signaling is not restricted to Y1 cells. Similar results were obtained with immortalized forebrain neurons that rapidly extend long neurites in response to isoproterenol, 8-bromo-cAMP, or forskolin.

Paxillin subserves intertwined scaffolding and signaling functions in focal adhesions via distinct domains that bind >6 effector proteins (16, 21, 22). Csk, Crk, and tensin contain SH2 domains that bind PY-containing segments of paxillin. Engagement of integrins with extracellular matrix stimulates (indirectly) tyrosine phosphorylation of paxillin, thereby enhancing multi-protein complex formation (16, 17). cAMP-controlled tyrosine dephosphorylation of paxillin would generate incomplete (possibly unstable) focal adhesion complexes and compromise signaling between integrins and effectors in the actin cytoskeleton. A logical speculation is that changes in the quality or intensity of such signals coupled with a co-operative weakening in focal adhesion structure due to the loss of SH2 binding sites results in the displacement of stress fibers from the cell cortex and the redistribution of F-actin.

In summary, our results suggest that a PTP and paxillin could be early, target/effecter proteins in a cAMP-controlled signaling pathway that regulates cell shape. Y1 cells provide a system for further evaluation of this proposition.

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**cAMP Regulates Paxillin Dephosphorylation and Cell Shape**

29215