Kalirin, a Multifunctional PAM COOH-terminal Domain Interactor Protein, Affects Cytoskeletal Organization and ACTH Secretion from AtT-20 Cells*

(Received for publication, June 11, 1998, and in revised form, November 19, 1998)

Richard E. Mains, M. Rashidul Alam, Richard C. Johnson, Daniel N. Darlington†, Nils Bäck§, Tracey A. Hand, and Betty A. Eipper¶

From the Departments of Neuroscience and Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, the †Departments of Surgery and Physiology, The University of Maryland Medical School, Baltimore, Maryland 21201, and the §Institute of Biomedicine, Department of Anatomy, University of Helsinki, Helsinki, Finland 00014

The production and regulated secretion of bioactive peptides require a series of lumenal enzymes to convert inactive precursors into bioactive peptides plus several cytosolic proteins to govern granule formation, maturation, translocation, and exocytosis. Peptidylglycine α-amidating monooxygenase (PAM), an enzyme essential for biosynthesis of many peptides, is an integral membrane protein with trafficking information in both its lumenal and cytosolic domains. Kalirin, a PAM cytosolic domain interactor protein with spectrin-like repeats and GDP/GTP exchange factor activity for Rac1, is expressed with PAM in neurons but is not expressed in the anterior pituitary or AtT-20 corticotrope cells. Expression of Kalirin alters the cytoskeletal organization of Chinese hamster ovary and AtT-20 cells expressing membrane PAM. Expression of membrane PAM also alters cytoskeletal organization, demonstrating the presence of endogenous proteins that can mediate this effect. Significant amounts of both PAM and Kalirin fractionate with cytoskeletal elements. Since cytoskeletal organization is critical for exocytosis, constitutive-like and regulated secretions were evaluated. Whereas the constitutive-like secretion of adrenocorticotrophic hormone (ACTH) is increased by expression of membrane PAM, regulated secretion is eliminated. Expression of Kalirin in AtT-20 cells expressing membrane PAM restores stimulated secretion of ACTH. Thus, Kalirin or its homologue may be essential for regulated secretion, and the PAM-Kalirin interaction may coordinate intragranular with cytosolic events.

Peptidylglycine α-amidating monooxygenase (PAM)1 (EC 1.14.17.3) is the only enzyme known to produce α-amidated peptides (1–7). It is an integral membrane protein whose trafficking to the appropriate subcellular compartments depends upon interactions between its COOH-terminal domain and cytosolic proteins (8, 9). Truncation of the COOH-terminal domain of PAM immediately following its single transmembrane domain yields active bifunctional enzyme that accumulates on the plasma membrane, unable to adopt its normal trans-Golgi network and secretory granule localization or to undergo endocytosis (9).

Kalirin, a novel member of the Dbl family of GDP/GTP exchange factors, was identified as a protein that interacts with routing determinants in the COOH-terminal cytosolic domain of membrane PAM (10, 11). The 200-kDa Kalirin protein consists of 9 spectrin-like repeats, a Dbl homology (DH) domain followed by a pleckstrin homology domain, an SH3 domain, and a COOH-terminal domain (Fig. 1). The region of Kalirin that interacts with PAM is contained within the spectrin-like repeats. The DH domain is typical of GDP/GTP exchange factors specific for members of the Rho subfamily of small GTP-binding proteins, and Kalirin interacts with Rac1 (11). Pleckstrin homology domains often support membrane localization through interactions with phospholipids (12–16), and SH3 domains mediate interactions with Pro-rich sequences in target proteins (17–20).

PAM is expressed in a wide variety of cell types, including neuronal, endocrine, glial, and endothelial cells (1–7). The disposition of amidated peptide products differs with cell type, with polarized secretion from neuronal processes, prolonged storage and tightly regulated release from pituitary cells, and rapid secretion from endothelial cells and glia (21–24). The identification of PAM interactors whose expression is limited to a subset of the PAM-producing cells was thus anticipated. Consistent with this, our initial studies indicated that Kalirin expression was high in the central nervous system but low in anterior pituitary and heart atrium, tissues in which PAM is highly expressed (10, 11, 25).

Anticipating that the cell type selected would affect the responses observed, we sought an appropriate system in which to investigate the mechanisms through which Kalirin affects the trafficking of PAM. For example, in non-neuroendocrine cells, Rac1 plays a role in membrane ruffling (13, 15, 26–29), whereas in neurons, axonal growth is affected (29–32). AtT-20 corticotrope tumor cells were explored because they exhibit regulated release of ACTH and express a number of neuronal markers (33). In addition, our earlier studies demonstrated that overexpression of Kalirin in AtT-20 cells caused extension of long, branched neuritic processes and accelerated the cleavage of membrane PAM (11).

We first established that PAM and Kalirin expression are essentially coincident in the adult rat central nervous system. We next determined that AtT-20 cells, like anterior pituitary corticotropes, lack significant levels of Kalirin but do express a
related Dbl family member, Trio (34) (Fig. 1). After using a non-neuroendocrine cell line to establish the ability of Kalirin to alter the organization of the actin cytoskeleton, we explored the hypothesis that expression of Kalirin in AtT-20 cells could also alter the actin cytoskeleton. We next explored the hypothesis that expression of PAM-1, acting through endogenous proteins, might also alter cytoskeletal organization. Finally, we evaluated the functional consequences of this altered cytoskeletal organization by measuring ACTH secretion from cells expressing Kalirin and PAM-1.

MATERIALS AND METHODS

Northern Blot Analysis—Poly(A)+ RNA (1 µg) was fractionated on denaturing agarose gels, transferred to nylon membranes, and visualized after hybridization with radiolabeled cDNA probes specific for the DH domain of rat Kalirin (nt 3790–4635) or rat Trio (nt 3925–4199, using human Trio numbering) (34). The blots were stripped and reprobed using radiolabeled cDNA probes specific for the spectrin-like domains of rat Kalirin (nt 1364–2600) or rat Trio (nt 1335–2642). A probe specific for ribosomal protein S26 was used for normalization (35). RNA loading was compared using the ribosomal protein S26 (35). All of the blots shown for Kalirin and Trio were exposed for the same amount of time and used probes of similar specific activity. Similar data were obtained from three sets of Northern analyses.

Growth and Analysis of AtT-20 Cells—AtT-20/D16v cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum (HyClone, Logan, UT) and 10% NuSerum (Collaborative Research, Bedford, MA) and passaged weekly. Establishment of cell lines stably expressing both PAM-1 and Myc-Kalirin or Kalirin was described previously (11). CHO cell lines stably expressing Myc-Kalirin were established as described for AtT-20 cells (11).

Electron Microscopy—Cells were fixed with 2.5% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M phosphate buffer, pH 7.2, for 30 min, postfixed with 1% osmium tetroxide for 60 min, dehydrated, and embedded in an Epoxy resin. Inverted Beem capsules with the bottom cut off were placed over selected areas of the cultures, and after polymerization overnight the capsules were filled with resin and polymerized for an additional 24 h. The bottom of the culture vessel was broken off, and the cells were sectioned in the horizontal plane. The sections were poststained with uranyl acetate and lead citrate and photographed with a Jeol 1008 electron microscope.

Dual Immunostaining in Situ Hybridization—Adult male rats were deeply anesthetized and fixed by perfusion (10, 11, 40). Frozen sections were processed for in situ hybridization for Kalirin and immunocytochemistry for PAM as described (10, 11, 40).

Immunocytochemical Procedures—AtT-20 cells plated onto polylysine-coated chamber slides were fixed with ice-cold methanol or warm 4% paraformaldehyde and stained as described previously (9, 41). Dual staining used a rabbit polyclonal antiserum for Kalirin (Ab2581, 1:1000) and a mouse monoclonal antibody for γ-adaptin (Transduction Laboratories, Lexington, KY). The rabbit polyclonal antibody was visualized with FITC-tagged goat anti-rabbit F(ab)2 IgG (H+L) (Caltag Laboratories, Burlingame, CA), and the monoclonal antibody was visualized with Cy5S9I-tagged AffiniPure donkey anti-mouse IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (41). Antibody uptake experiments with AtT-20 cells have been described previously (9). Actin filaments were visualized in AtT-20 and CHO cells fixed in 3.7% paraformaldehyde for 10 to 30 min using FITC-hyaluronidase (0.125 to 0.5 µg/ml) (13, 27, 42, 43). Cells were viewed under epifluorescence optics with a Zeiss Axioskop microscope and FITC (BP 485/20, barrier filter 520–560) and rhodamine (BP 546/12, LP 590) filters (Carl Zeiss, Inc., Thornwood, NY) and photographed using a Princeton In-
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RESULTS

Kalirin Is Highly Expressed in Central Nervous System Neurons That Express PAM—The paired use of in situ hybridization and immunocytochemistry has allowed us to examine the presence of Kalirin and PAM in neurons in various regions of the brain. We had previously established that Kalirin and PAM could be found in the same neurons (11), but a more extensive analysis shows that there is a virtual identity of PAM and Kalirin expression in the adult rat central nervous system (Fig. 2). We could not find significant numbers of PAM-expressing neurons that lacked Kalirin nor could we find Kalirin-expressing neurons that lacked PAM. In certain regions of the adult rat brain, such as cerebellum, both PAM and Kalirin transcripts are undetectable (11, 44). By contrast, in other regions of the brain (e.g., the hippocampus; olfactory bulb; hypothalamus; cingulate, piriform, and parietal regions of the cortex; frontal cortex), a very high percentage of the neurons was positive for both PAM and Kalirin. In the pyramidal cell layer of the hippocampus (Fig. 2D), and among the magnocellular (neuroendocrine) neurons of the hypothalamus, all of the neurons were positive for PAM and Kalirin. Peptide-containing neurons, as marked by PAM expression in previous studies (44), were very abundant in several parts of the central nervous system.

Although Northern blot analysis indicated that pituitary was not a major site of Kalirin expression (11), we used in situ hybridization to determine whether a few Kalirin-expressing cells could be identified among a majority of non-expressing cells (Fig. 2B). We failed to find any evidence for Kalirin expressing cells in the pituitary. Thus expression of Kalirin in the adult rat appears to be limited to the central nervous system.

Kalirin Is Not Highly Expressed in AtT-20 Cells—The fact that Kalirin is not highly expressed in the anterior pituitary suggests that Kalirin, per se, is not essential for PAM to function as a peptide a-amidating enzyme nor for the formation and storage of large dense core vesicles. Since AtT-20 cells are derived from anterior pituitary corticotropes and store products derived from proopiomelanocortin (POMC) in large dense core vesicles, we evaluated the expression of Kalirin in this cell line. As observed for rat anterior pituitary, Kalirin mRNA was not detectable when AtT-20 cells were examined (Fig. 1). In contrast, rat cortex yielded a robust signal with several different forms of Kalirin mRNA. Trio was examined because it is the closest known homologue of Kalirin; a single 12-kilobase pair Trio transcript was apparent in both anterior pituitary and AtT-20 cells. Trio transcripts are expressed at similar levels in anterior pituitary and cortex.

Kalirin Alters the Actin Cytoskeleton in Non-neuroendocrine Cells—Since Kalirin contains spectrin-like domains and is a guanine nucleotide exchange factor for Rac1 (45), we wanted to determine whether its expression would affect the actin cytoskeleton. The effects of Dbl family members on cytoskeletal organization are routinely examined in non-neuroendocrine cells, so we expressed Kalirin in CHO cells (Fig. 3). CHO cells expressing Kalirin exhibited a morphology substantially different from that of nontransfected CHO cells; they were more rounded, making less contact with the culture dish, and spike-like structures could be seen extending from their surface.

Staining of nontransfected CHO cells with FITC-phalloidin, a mushroom toxin that binds selectively to filamentous actin, revealed a highly ordered array of stress fibers extending from one margin of the cell to the other; nontransfected CHO cells were thin, exhibiting extensive contact with the substrate (Fig. 3). Stress fibers were less prevalent in the CHO cells expressing Kalirin; instead, isolated patches of filamentous actin could be seen near the cell surface.

Exogenous Kalirin Is Localized to the Golgi Region of AtT-20 Cells—Our previous studies demonstrated that expression of Kalirin in AtT-20 cells expressing exogenous membrane PAM altered the morphology of the cells and increased the speed with which PAM underwent endoproteolytic cleavage within the regulated secretory pathway (11). At the time the original studies were performed, our antisera did not allow us to determine the subcellular localization of exogenous Kalirin expressed in AtT-20 cells. By using two new rabbit polyclonal...
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FIG. 3. Expression of Kalirin alters cytoskeletal organization in CHO cells. Nontransfected (nontrans) CHO cells and CHO cells expressing Myc-Kalirin were fixed in paraformaldehyde for 10 min, permeabilized, and exposed to FITC-phalloidin for 30 min. Cells were photographed under identical conditions. Spike-like projections in Kalirin-expressing cells are marked (*).

Expression of Kalirin and/or PAM Alters the Cytoskeleton in AtT-20 Cells—We next evaluated the effect of Kalirin on the actin cytoskeleton. Staining nontransfected AtT-20 cells with FITC-phalloidin revealed a fairly disordered pattern of actin filaments; some staining was apparent at the margins of the cells, and speckles of staining were apparent throughout much of the cytoplasm (Fig. 5).

Although AtT-20 cells produce fully amidated product peptides from POMC (46, 47), they express PAM at a level no more than 10% that observed in the anterior pituitary. It has not yet proven possible to generate ACT-20 cell lines that overexpress full-length Kalirin unless the cells are also expressing exogenous PAM. Therefore an AtT-20 cell line expressing a Kalirin fragment (Kalirin-(447–1124)) comprised only of spectrin-like repeats was examined. We then asked whether expression of this Kalirin fragment affected cytoskeletal organization in AtT-20 cells (Fig. 5). AtT-20 cells expressing Kalirin-(447–1124) exhibited a filamentous actin staining pattern that was much more localized to the Golgi region than in non-transfected cells; the speckled staining observed throughout the cytoplasm of non-transfected AtT-20 cells was absent.

If PAM-1 interacts with endogenous proteins that resemble Kalirin, expression of PAM-1 might also be predicted to alter the actin cytoskeleton. Consistent with this prediction, the filamentous actin in AtT-20 PAM-1 cells was more localized to the region of the Golgi than in non-transfected cells (Fig. 5). Thus AtT-20 cells may contain an endogenous Kalirin-like protein.

Expression of PAM and Kalirin together resulted in more intense staining for filamentous actin at the margins of the cell; in addition, spike-like protrusions staining intensely for filamentous actin were apparent on many cells expressing both PAM-1 and Kalirin (marked with an asterisk). The remainder of the filamentous actin was localized to the Golgi region of the cell; this same region of the cell contains most of the membrane PAM. Staining in the sub-plasma membrane region was not as intense in AtT-20 PAM-1 cells as in cells expressing both Kalirin and PAM-1.

Electron microscopy was used to compare the structure of nontransfected AtT-20 cells to AtT-20 cells expressing PAM-1 or PAM-1 and Myc-Kalirin (Fig. 6). Non-transfected AtT-20 cells showed scattered microtubules in the cytoplasm and microtubules and a few ordered filaments in the cell processes. Bundles of actin filaments were seen at the tips of the cell processes (Fig. 6A, dark arrows). Cortical actin filaments in the sub-plasma membrane region could occasionally be visualized. In cells expressing PAM-1, as well as cells expressing PAM-1 plus Kalirin, thick bundles of cortical actin filaments could occasionally be observed (dark arrows). In addition, bundles and large aggregates of intermediate filaments were seen in cell processes (Fig. 6, B and C, asterisks). In some cells, similar bundles were seen near the nucleus (Fig. 6C, c). Long filament-containing microspikes are seen at the left in Fig. 6B, the ultrastructural equivalent of the fine fluorescent processes visible using FITC-phalloidin (Fig. 5, asterisks). It is expected that filamentous actin, microtubules, and intermediate filaments will be found together in cells, but normally intermediate filaments are restricted to nerve fibers entering the pituitary and are not abundant in pituitary endocrine cells (48). The ultrastructural observations thus indicate a widespread effect of PAM-1 expression on the cytoskeleton, affecting the distribution of both actin filaments and intermediate filaments.

PAM and Kalirin Are Associated with the Cytoskeleton in AtT-20 Cells—To investigate these interactions among PAM, Kalirin, and cytoskeletal elements in more detail, AtT-20 cells expressing Kalirin and membrane PAM were fractionated using standard conditions known to preserve many cytoskeletal interactions and keep intermediate filaments intact (15) (Fig. 7). Lysates prepared by incubating cells in isotonic buffer containing detergent were separated into a low speed cytoskeletal fraction, a high speed cytoskeletal fraction, and a soluble fraction. The distributions of PAM and Kalirin were compared following Western blot analysis. The soluble 45-kDa PHM protein appeared entirely in the soluble fraction, indicating that membranes were adequately disrupted. Integral membrane forms of PAM (PAM-1 and PALm) were recovered primarily in the soluble fraction, but a significant fraction was recovered in the high speed cytoskeletal fraction.

When the distribution of Kalirin was evaluated, it was apparent that Kalirin was recovered with PAM in the high speed cytoskeletal pellet and the soluble fraction (Fig. 7). A cross-reactive endogenous protein of 170 kDa exhibited a similar fractionation pattern. It is not yet clear whether this cross-reactive protein represents the endogenous functional equivalent of Kalirin, although the Western signals are blocked by preincubation of the antisera with purified Kalirin4/7. Actin
was distributed approximately equally among the low speed cytoskeletal fraction, the high speed cytoskeletal fraction, and the supernatant (data not shown). Tiam 1, a neuronally expressed member of the Dbl family of proteins, exhibited a similar distribution pattern upon fractionation with the same cytoskeletal buffers (15).

Expression of Kalirin Alters Secretion of ACTH-related Peptides—The fact that expression of PAM and Kalirin had a striking effect on the cytoskeletal system suggested that expression of PAM and Kalirin might affect the secretory pathway. For example, slight disruption of the actin cytoskeleton in pancreatic acinar cells resulted in a pattern of protein secretion paralleling regulated exocytosis, even in the absence of Ca\(^{2+}\) (49).

To test this hypothesis, basal and stimulated secretion of proopiomelanocortin-derived peptides were examined in AtT-20 cells stably expressing membrane PAM, a fragment of Kalirin, or both membrane PAM and full-length Kalirin. Regulated secretion from AtT-20 cells was studied by applying corticotropin-releasing hormone or BaCl\(_2\). The radioimmunoassay used for these studies is specific for the COOH terminus of ACTH (Fig. 8) (37–39). Since the assay recognizes only POMC products that have undergone at least one neuroendocrine-specific endoproteolytic cleavage, this assay measures constitutive-like and regulated secretion, usually attributed to secretion from immature and mature large dense core vesicles, respectively (50–53).

Stimulation of nontransfected AtT-20 cells resulted in a 3-fold increase in secretion rate, as expected from past work (37, 38, 54–56). Expression of membrane PAM raised the basal rate of ACTH secretion 3-fold (\(p < 0.012\) compared with nontransfected) and obliterated any significant stimulation of secretion by corticotropin-releasing hormone or BaCl\(_2\) (Fig. 8) or by phorbol esters (22). Expression of Kalirin-(447–1124), the PAM interactor domain of Kalirin, resulted in nearly a 2-fold increase in the basal rate of ACTH secretion compared with nontransfected cells (\(p < 0.02\)). Secretion of ACTH from cells expressing Kalirin-(447–1124) was still increased in response to secretagogues.
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Cells expressing only membrane PAM exhibited increased basal secretion when compared with non-transfected cells and failed to respond to secretagogue. Cells expressing both membrane PAM and Kalirin showed a doubling in their basal rate of secretion compared with cells expressing only membrane PAM \((p < 0.002)\) and a 5-fold increase in their basal rate of secretion compared with nontransfected cells \((p < 0.001)\). In contrast to cells expressing only membrane PAM, cells expressing both membrane PAM and Kalirin showed a 3-fold stimulation of ACTH release in response to secretagogue; fully 30\% of the cell content of ACTH was released within a single 30-min period of exposure to secretagogue. Two separate Kalirin lines and two separate PAM-1/Kalirin lines were examined, and each pair gave a similar response.

Expression of Kalirin Alters PAM/Antibody Internalization by AtT-20 Cells—Although localized primarily to the trans-Golgi network region of the cell at steady state, membrane PAM appears transiently on the cell surface; biochemical assays and antibody internalization studies indicate that membrane PAM that reaches the cell surface is rapidly internalized and returned to a late endocytic compartment \((8, 9, 57)\). An increase in basal secretion such as that observed upon expression of Kalirin in AtT-20 cells expressing PAM-1 would be expected to alter the rate of delivery of membrane PAM to the cell surface and result in alterations in the internalization of PAM from the cell surface.

In order to test this hypothesis, internalization of membrane PAM from the cell surface was visualized by incubating live cells with an antibody to PAL (part of the ectodomain of this type I integral membrane protein) and then in medium lacking antibody. Antibody bound to PAM is internalized with PAM and visualized with a FITC-tagged secondary antibody. Expression of Kalirin significantly increased internalization of PAM/antibody from the cell surface (Fig. 9). When examined only 5 min after incubation with antibody, the PAM-antibody complex in membrane PAM cells was largely collected in the perinuclear region; very little staining was observed in cell processes. In contrast, the PAM-antibody complex internalized by cells expressing Kalirin was present in cell processes, and much of the PAM-antibody complex had not been collected in the perinuclear region.

After a 30-min chase period the PAM-antibody complex has been collected in a fairly compact region near the nucleus of cells expressing only membrane PAM; very little punctate staining was observed at the margins of the cell or in cell processes. In contrast, PAM-antibody complex internalized by AtT-20 cells expressing both PAM and Kalirin was still readily detected in cell processes and throughout the cytosol, in addition to some concentration in the perinuclear region. In addition, the overall intensity of the signal was substantially higher in cells expressing both PAM and Kalirin.

**DISCUSSION**

PAM proteins catalyze peptide \(\alpha\)-amidation in the trans-Golgi network and immature secretory granules but can also be identified at the cell surface and in various endocytic structures \((8, 39, 41)\). The cytosolic domain of PAM contains much of the routing information that determines the complex itinerary taken by this protein. Phosphorylation and dephosphorylation of PAM clearly play important roles, and it seems likely that
PAM interacts with a series of cytosolic proteins in order to traverse multiple subcellular compartments (58). The first three PAM cytosolic domain interactor proteins identified include Kalirin, a protein kinase (P-CIP2) and a novel protein (P-CIP1) (10, 11, 59). We used endocrine cells making bioactive peptides and P-CIP2 as a model system in which to test the hypothesis that Kalirin acts in part through its interactions with the cytoskeleton.

Expression of Kalirin is largely restricted to the central nervous system (11, 60). There is a one-to-one correspondence of PAM and Kalirin expression in central nervous system neurons, and in several regions virtually all neurons express both PAM and Kalirin (Fig. 2). In contrast, the endocrine cells of the anterior pituitary express high levels of PAM without expressing significant amounts of Kalirin (Fig. 1). Like the cells of the anterior pituitary, AtT-20 corticotrope tumor cells do not express high levels of Kalirin. Trio (34), a close homologue of Kalirin, is found in pituitary, but it is unclear whether Trio serves as a functional replacement for Kalirin in this tissue.

The occurrence of tissue-specific factors affecting large dense core vesicle membrane proteins was anticipated. Neurons, with their axonal, dendritic, and cell soma regions, must target large dense core vesicles to specific release sites. In Aplysia and in Lymnaea, distinct classes of large dense core vesicles containing different products of pro-ELH processing are released into the circulation from axon endings or into extracellular space from collaterals (61–63). The large dense core vesicles of atrial myocytes are localized to the perinuclear region (64, 65), whereas corticotrope vesicles accumulate under the plasma membrane (39, 66, 67), and the vesicles in hypothalamic neurosecretory neurons are transported long distances to terminals in the neural lobe of the pituitary.
Kalirin affects organization and secretion from AtT-20 cells. Nontransfected cells are assumed to express an endogenous Kalirin-like molecule, which we propose is required for regulated Ca\(^{2+}\)-dependent secretion of ACTH, possibly via interactions with Rac1 (15) and the actin cytoskeleton. Overexpression of integral membrane PAM enhances constitutive-like secretion and inhibits regulated secretion, perhaps by binding the endogenous Kalirin-like molecule. Expression of exogenous Kalirin then restores the stimulatability of ACTH secretion. The exogenous Kalirin, which localizes in the Golgi region, also enhances access of newly synthesized PAM to endoproteases in immature secretory granules and affects the internalization of PAM from the cell surface.

We were unable to generate AtT-20 cell lines stably expressing full-length Kalirin. Kalirin could, however, be expressed in AtT-20 cells stably overexpressing PAM-1 at levels found in the anterior pituitary. Kalirin was concentrated in a broad area that encompassed the trans-Golgi network, the site of secretory granule formation (Fig. 4). A significant amount of the Kalirin and the membrane PAM fractionated with the cytoskeleton (Fig. 7). As predicted by its ability to interact with Rac1 (11), Kalirin expression led to a marked rearrangement of the actin cytoskeleton in both non-neuroendocrine and neuroendocrine cells (Figs. 3 and 5). The differences in the morphological responses observed in CHO cells and AtT-20 cells emphasize the importance of endogenous proteins in determining the response of a cell to expression of Kalirin. AtT-20 cells expressing both PAM and Kalirin produced longer and more highly branched neuritic processes (11), whereas CHO cells expressing Kalirin rounded up and extended short, spike-like projections (Fig. 3).

The fact that Kalirin expression altered cytoskeletal organization led us to ask whether expression of membrane PAM might also affect the cytoskeleton. Based on staining with FITC-phalloidin and on ultrastructural analysis, expression of membrane PAM in the absence of exogenous Kalirin led to a marked rearrangement of both the actin and intermediate filament components of the cytoskeleton (Figs. 5 and 6). Patches of filamentous actin were localized to the region of the trans-Golgi network instead of being distributed throughout the cytosol. Bundles of intermediate filaments became prevalent in cell processes. These observations suggest that AtT-20 cells express a protein or set of proteins that mediate the interaction of PAM with cytoskeletal elements. Our working model for the effects of membrane PAM and Kalirin in AtT-20 cells is presented in Fig. 10. Although we postulate the involvement of an endogenous Kalirin-like protein, other proteins such as P-CIP2, the protein kinase identified through its ability to interact with the CD of PAM, could also mediate some of the effects of PAM on the cytoskeleton (10, 68, 69) (Fig. 10). For example, by phosphorylating stathmin or SCG10, P-CIP2 could affect microtubule stability (68, 69).

We previously demonstrated that expression of Kalirin resulted in more rapid cleavage of membrane PAM to produce soluble PHM and membrane PAL, suggesting a role for Kalirin in the formation of immature secretory granules (11). In this study we noted an alteration in the behavior of PAM-antibody complexes following internalization from the plasma membrane (Fig. 9). In cells expressing Kalirin, the internalized PAM-antibody complexes fail to accumulate in a compact, perinuclear region and instead remain dispersed throughout the cell. Both the effect of Kalirin on the trafficking of newly synthesized PAM exiting the trans-Golgi network and on plasma membrane PAM undergoing endocytosis may involve its effects on the actin cytoskeleton (70) (Fig. 10).

It is clear from studies on lactotropes (42) and pancreatic acinar cells (49) that precise control of the actin cytoskeleton is essential for normal basal and stimulated secretion. Overexpression of membrane PAM, Kalirin-(447–1124), or full-length Kalirin along with membrane PAM caused enhanced constitutive-like secretion of ACTH (Fig. 10). The fact that Kalirin-(447–1124) exerts this effect indicates that the GDP/GTP exchange factor domain is not essential for the effect of Kalirin on constitutive-like secretion. Kalirin-mediated interactions with endogenous proteins such as HAP1 (60), a cytosolic protein that interacts with p150\(^{Glu}\), might play a role. The p150\(^{Glu}\) protein is an accessory protein for cytoplasmic dynein, which participates in microtubule-dependent retrograde transport of membranous organelles (71, 72). Endogenous proteins such as P-CIP2 may also play a role in this response.

In AtT-20 lines expressing membrane PAM, secretagogue treatment failed to stimulate ACTH secretion (22). Upon expression of Kalirin, application of secretagogue resulted in a robust stimulation of ACTH secretion (Fig. 8); fully 30% of the cellular content of ACTH was released within a 30-min period. Increased efficacy of regulated secretion upon expression of Kalirin has been seen in three AtT-20 cell lines with independent Kalirin-related constructs, suggesting that the exogenous Kalirin interacts with endogenous AtT-20 proteins in the restoration of regulated secretion. Nontransfected cells are postulated to express an endogenous Kalirin-like molecule, which is required for regulated Ca\(^{2+}\)-dependent secretion of ACTH (Fig. 10). Overexpression of integral membrane PAM would bind the

**Fig. 10.** Model for the effects of integral membrane PAM and Kalirin on ACTH secretion. Nontransfected cells are assumed to express an endogenous Kalirin-like molecule, which we propose is required for regulated Ca\(^{2+}\)-dependent secretion of ACTH, possibly via interactions with Rac1 (15) and the actin cytoskeleton. Overexpression of integral membrane PAM enhances constitutive-like secretion and inhibits regulated secretion, perhaps by binding the endogenous Kalirin-like molecule. Expression of exogenous Kalirin then restores the stimulatability of ACTH secretion. The exogenous Kalirin, which localizes in the Golgi region, also enhances access of newly synthesized PAM to endoproteases in immature secretory granules and affects the internalization of PAM from the cell surface.
enogenous Kalirin-like molecule and remove it from the site of regulated Ca\(^{2+}\)-dependent secretion, thereby inhibiting regulated secretion. Expression of exogenous Kalirin would then restore the stimulatability of ACTH secretion. Kalirin-(447–1124) failed to enhance regulated secretion of ACTH, suggesting an essential role for the GDP/GTP exchange factor domain of Kalirin in regulated exocytosis. The ability of added Kalirin to enhance the action of BaCl\(_2\) places the step(s) affected by Kalirin downstream of receptor-mediated signaling events initiated by secretagogues such as corticotropin-releasing hormone or pharmacological agents such as phorbol esters.

The observation that an integral membrane protein involved in peptide biosynthesis (PAM) alters the pattern of peptide secretion and the organization of the cytoskeleton by interacting with a Db1 family member is unprecedented. Many members of the Db1 family were first identified as oncogenes, and their normal functions are still under investigation (73). Trio, a member of the Dbl family of proteins, is involved in pheromone-mediated arrest of growth, activation of transcription, polar-}

Acknowledgments—Dr. Michel Streuli (Division of Tumor Immunology, Dana-Farber Cancer Institute) generously provided a 2.4-kilobase pair cDNA fragment of human Trio. We thank Dr. Sue Craig (Johns Hopkins University) for advice on the cytoskeleton. We thank Cathy Caldwell for assistance in cell culture and Marie Bell for general laboratory assistance.

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