A Regulatory Role for cAMP-dependent Protein Kinase in Protein Traffic along the Exocytic Route*

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The influence of protein kinase A activity on transport of newly synthesized vesicular stomatitis virus G glycoprotein along the exocytic pathway was examined. Transport of vesicular stomatitis virus G glycoprotein to the cell surface was inhibited by N-[2-(p-bromocinnamylamino)ethyl]-5-isooquinolinesulfonamide (H-89), a selective inhibitor of protein kinase A. This block occurred at the exit of the Golgi complex, whereas transport through the Golgi compartments or from the endoplasmic reticulum to the Golgi was decreased in the presence of H-89. As judged by immunofluorescence microscopy, endoplasmic reticulum to Golgi transport was accelerated in cells incubated with activators of protein kinase A such as isobutylmethylxanthine (IBMX) or forskolin (FK). Treatment with IBMX and FK also increased transport from the trans-Golgi network to the cell surface. During incubation with IBMX and FK, the organization of the Golgi complex was altered showing intercisternal fusion and miscompartmentalization of resident proteins. These structural changes affected both the kinetics of acquisition of endoglycosidase H resistance and transport activities. These data support a differential regulatory role for protein kinase A in different transport steps along the exocytic pathway. In particular, transport from the trans-Golgi network to the cell surface was dependent on protein kinase A activity. In addition, the results suggest the involvement of this enzyme on the maintenance of the Golgi complex organization.

Recent evidence suggests a regulatory role for signal transduction molecules in vesicle-mediated transport along both the exocytic and endocytic pathways. Several heterotrimeric G proteins have been implicated in different steps of membrane trafficking (1, 2). Second messengers and protein kinases have also been shown to influence various transport processes. For instance, cAMP and protein kinase A activity have been reported to stimulate apical directed transcytosis and secretion in epithelial cells (3–6). Protein kinase A seems to exert a differential regulatory role in polarized cells. Transport from the trans-Golgi network (TGN) to the apical surface but not to the basolateral surface was stimulated by agents that activate protein kinase A (6, 7). Phorbol esters and protein kinase C have also been implicated in transport regulation. Association of ADP-ribosylation factor and coatomer proteins to Golgi membranes was increased by treatment with agents that activate protein kinase C (8). Constitutive transport from the TGN to the cell surface, regulated secretion, apical exocytosis, and transcytosis have all been shown to be modulated by protein kinase C activity (7, 9–12). Together, these studies extend previous observations on the requirement for protein phosphorylation in certain transport steps such as protein transfer from the endoplasmic reticulum (ER) to the Golgi complex (13), formation of secretory vesicles from the TGN (14), and endosome fusion (15). Particular isoforms of both protein kinases A and C have been localized in the ER and the Golgi complex (16–19). It is unknown, however, in what way these kinases modulate either organelle structure and/or transport processes.

Other evidences indicate an essential role for protein kinases in particular transport steps. Transport of hydrolases from the TGN to either the yeast vacuole or mammalian lysosomes requires phosphatidylinositol 3-kinase activity (13, 20, 21). Therefore, the available evidences indicate that different protein kinases regulate membrane trafficking in eukaryotic cells although the importance and extent of such control is unclear at present. In this study we have investigated the role of cAMP and protein kinase A activity on transport along the entire exocytic pathway. We studied the constitutive transfer of an integral membrane glycoprotein, vesicular stomatitis virus (VSV)-G protein, from the ER through the Golgi to the cell surface. The results indicate a stimulatory role for protein kinase A on different steps along this route and emphasize the influence of this activity on Golgi structural organization.

EXPERIMENTAL PROCEDURES

Materials—The tsO45 mutant of VSV was obtained from Dr. W. Balch (Scripps Research Institute, La Jolla, CA). 3-Isobutyl-1-methylxanthine (IBMX), forskolin (FK), N\,\,p\,\,2\,-\,O\,-\,dibutyryladenosine\,-\,3\,\,-\,5\,-\,cylic monophosphate (dibutyryl-cAMP), 8-bromoadenosine-3'-5'-cyclic monophosphate (8-Br-cAMP), protein A, protein G-Sepharose, cycloheximide, type VIII neureminidase, saponin, and bovine serum albumin were purchased from Sigma. Sp-5,6-dichloro-cBIMPS (Sp-cBIMPS) was from Biolog (Germany); endoglycosidase H (endo H) was from Boehringer Mannheim (Germany); N-[2-(p-bromocinnamylamino)ethyl]-5-isooquinolinesulfonamide (H-89) and cholerae toxin were from Calbiochem. Tissue culture reagents were purchased from Life Technologies, Inc.

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Inc., and Tran^35S-label and 125I-labeled epidermal growth factor were from ICN Biochemicals (Cleveland, OH). P5D4 monoclonal antibody against the carboxyl terminus of VSV-G protein (22) was either a gift of Dr. T. Kreis (University of Geneva, Switzerland) or purchased from Sigma. Monoclonal antibody 8G5F11 (clone I1) specific for the extracytoplasmic domain of VSV-G (23) was kindly provided by Dr. D. S. Lyles (Wake Forest University, NC). Polyclonal antibodies recognizing α1,2-mannosidase II (24) and TGN-38 (25) were generously provided by Drs. M. G. Paraguari (University of California San Diego) and J. P. Luzio (University of Cambridge, UK), respectively. Fluorescein- and rhodamine-labeled secondary antibodies were purchased from TAGO (Burlingame, CA).

Cell Culture and Virus Infection—Monolayers of normal rat kidney (NRK) cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Confluent cultures were infected at 32 °C with 10–20 plaque-forming units/cell of the tsO45-VSV strain as described (13). Virus was propagated in COS cells. Metabolic Labeling and Transport Incubations—Cells cultured on 100-mm dishes were used. 3 h postinfection at the permissive temperature (32 °C) they were trypsinized and transferred to the restrictive temperature (39.5 °C), and chased for 40 min at the permissive temperature (32 °C) in the presence of the indicated concentrations of H-89. VSV-G protein was immunoprecipitated from both the cell surface and the extracellular virions and subjected to SDS-PAGE analysis. The amount of VSV-G protein reaching the cell surface was calculated. Values were normalized for the total amount of VSV-G protein present in the cells and expressed as percentage of VSV-G protein transported in untreated, control cells. The cellular ATP level was determined as described under “Experimental Procedures” and expressed as percentage of value in control cells.

Protein Assay—Cells were rinsed twice in PBS and then incubated at 100 °C for 5 min in 200 μl Tris, 20 mM MgSO_4, pH 7.7. Cell extracts were incubated at room temperature with a preparation of luciferin/luciferase obtained according to Strehler (29). Luminescence was measured in a luminometer and used to calculate ATP content by comparison with standard amounts of ATP.

RESULTS

Exocytic Transport of VSV-G Protein Is Inhibited by H-89—The isooquinolinesulfonamide known as H-89 was used as a selective protein kinase A inhibitor with almost no effect on other kinase families including protein kinases C and Ca^2+/calmodulin-dependent kinases. H-89 competes with ATP for binding to the catalytic subunits of protein kinase A (30). To study the role of protein kinase A on vesicle transport along the exocytic route, we examined the influence of H-89 on the intracellular transport of newly synthesized VSV-G, an integral membrane glycoprotein (31). NRK cells were infected with the tsO45 thermosensitive mutant of VSV, radiolabeled at the restrictive temperature (39.5 °C), and chased at the permissive temperature (32 °C) in the presence or absence of H-89. Transport was assessed by immunoprecipitation and SDS-PAGE analysis of G protein present at the cell surface and incorporated in free virions in the culture medium (Fig. 1). Transport was dramatically inhibited by H-89 in a dose-dependent manner with maximal inhibition occurring at 35–50 μM. Under these conditions only a few VSV-G molecules reached the cell surface. Importantly, this inhibition did not course with a decrease in the cellular level of ATP (Fig. 1), and cells incubated with H-89 remained viable after 1–2 h incubation. In addition, the integrity of the plasma membrane (as judged by trypan blue exclusion) and the ultrastructural organization of the ER (the TGN complex (Fig. 2)) remained unaltered during H-89 treatment. Therefore, these data suggested an inhibitory effect of H-89 on vesicle-mediated protein transport along the exocytic route.
H-89 Reversibly Blocks Transport from the TGN to the Cell Surface—The effects of H-89 treatment on transport of newly synthesized VSV-G protein were also evaluated by immunofluorescence. Infected cells were maintained at 39.5 °C for 4 h to allow for VSV-G protein to be concentrated in the ER while absent from other compartments in the secretory pathway. Cycloheximide was added to inhibit further synthesis, and transport out of the ER was analyzed at 32 °C in the presence or not of H-89 (Fig. 3). As expected VSV-G protein was not detected at the cell surface of cells treated for 2 h with H-89. Instead, it remained blocked in the Golgi complex showing colocalization with resident α-1,2-mannosidase II (Fig. 3A–A′). However, this block was fully reversible and VSV-G protein increasingly reached the cell surface after removal of the drug (Fig. 3B). The emerging idea was that H-89 selectively blocks exit of VSV-G protein from the Golgi complex and/or transport through the Golgi cisternae.

We therefore analyzed transport from the TGN to the cell surface. Cells were incubated at 19.5 °C for 1.5 h to accumulate radiolabeled VSV-G protein in the TGN (32). Transport was reestablished after shifting to the permissive temperature and evaluated by surface immunoprecipitation (Fig. 4A). In control cells arrival of VSV-G protein to the cell surface started after 10-min transport and increased during the following 30 min (Fig. 4B). In contrast, less than 10% of VSV-G molecules reached the surface of H-89-treated cells during the same time period (Fig. 4B). Taken together these results indicated that transport from the TGN to the cell surface was severely inhibited by H-89 and suggested the involvement of protein kinase A activity in the regulation of this transport step.

Influence of H-89 Treatment on Other Transport Steps—We studied the effects derived from incubating the cells with H-89 on transport of VSV-G protein through the early stations in the secretory pathway. Upon transfer to the Golgi complex VSV-G protein is converted to an endoglycosidase H (endo H)-resistant form that can be distinguished from the endo H-sensitive form present in the ER (33). In order to monitor transport from the ER to the Golgi, we evaluated the kinetics of acquisition of endo H resistance of newly synthesized VSV-G protein (Fig. 5A). In control, untreated cells conversion to an endo H-resistant form occurred with a half-life of 15 min including an initial lag period of 3–5 min (Fig. 5B). In cells exposed to H-89 the amount of VSV-G protein processed to an endo H-resistant form was significantly decreased; only 50% of the molecules were processed after a 60-min chase. In addition, the lag period was increased to almost 10 min (Fig. 5B). This suggested a reduced transport of VSV-G protein from the ER to the Golgi in H-89-treated cells.

We next extended these observations to the analysis of transport through the different Golgi cisternae. Infected, radiolabeled cells were incubated at 15 °C for 1.5 h to accumulate...
VSV-G protein at the ER/Golgi intermediate compartment (34, 35) and then chased at the permissive temperature. Processing of VSV-G protein to a form that is both endo H-resistant and neuraminidase-sensitive was taken as an indication of transport to a distal (trans/TGN) Golgi compartment (33). In control, untreated cells this occurred very rapidly and with no apparent lag; ~70% of VSV-G protein became neuraminidase-sensitive after 20-min transport (Fig. 6A). During this time less than 30% of VSV-G became neuraminidase-sensitive in cells incubated with H-89 (Fig. 6B). Therefore, ER to Golgi transport and transfer through the Golgi complex were both sensitive to H-89 treatment. This implied a regulatory role for protein kinase A in the control of these other transport events.

Effects of an Elevated Concentration of cAMP on VSV-G Exocytic Transport—Having seen the inhibitory effects of H-89 treatment on the intracellular transport of newly synthesized VSV-G protein, we examined the results derived of stimulus protein kinase A activity. We chose to activate protein kinase A with agents that increase the intracellular level of cAMP. IBMX was used as a specific inhibitor of cAMP-phosphodiesterase and forskolin (FK) as an activator of adenylate cyclase. Since in preliminary experiments a synergistic effect of both agents was observed, the cells were normally incubated with IBMX and FK simultaneously. By immunofluorescence transport from the ER to the Golgi was found to be stimulated in cells treated with IBMX + FK. Thus, VSV-G protein was localized in the perinuclear Golgi area as early as 5 min after shifting the cells to the permissive temperature while in control cells this took place after 8–10 min (Fig. 7, B and F). In fact, the clearance of the reticular, ER staining occurred faster in IBMX + FK-treated cells than in control cells (compare Fig. 7, C and G). Transport from the Golgi complex to the cell surface, on the other hand, occurred in both cases, but in cells incubated with IBMX + FK the Golgi staining persisted for a prolonged time (Fig. 7, D, and H). This was indicative of retention of VSV-G protein in the Golgi due to IBMX + FK treatment.

Structural Changes in Golgi Organization Caused by an Increase in cAMP—We noted important alterations in the ultrastructural organization of the Golgi complex during treatment with IBMX + FK (Fig. 8A). Instead of the typical pattern of stacked cisternae irregular membrane-bound compartments with fenestrations and associated vesicles were observed in the Golgi area. Golgi stacks that remained under these conditions did not show an ordered cisternal arrangement (Fig. 8A). This modification affected the entire Golgi stack as shown by the analysis of consecutive serial sections (Fig. 8, D–H). Apparently, within single Golgi stacks intercisternal fusion occurred during IBMX + FK incubation. In order to support this view we studied the relative distribution of two Golgi integral proteins detected by immunogold labeling. Both a–1,2-mannosidase II and TGN 38 which in control NRK cells localize to the middle Golgi cisternae (24) and the TGN region (25), respectively (Fig. 8B), were found colocalized in the same Golgi membranes after...
IBMX + FK treatment (Fig. 8C). More precisely, within the Golgi area 38% of the TGN 38 labeling and 31% of the mannosidase II labeling showed colocalization in IBMX + FK-treated cells. In contrast, less than 1% of the TGN 38 labeling and 8% of the mannosidase II labeling colocalized in control, untreated cells. We concluded that these agents gave rise to fusion events that affected different Golgi compartments causing mixing of previously segregated proteins. A similar modification of the Golgi complex was seen in cells exposed to cholerae toxin (Fig. 8, D–H) or incubated with cell-permeable cAMP analogs such as dibutyryl-cAMP or 8-Br-cAMP (not shown). This indicated that changes in Golgi organization were derived from a direct increase in the intracellular concentration of cAMP and did not depend on the agent giving rise to such an increase.

**Transport Activities in the Presence of an Elevated Concentration of cAMP**—Golgi structural changes induced by an increased level of cAMP seemed to affect the ability of this organelle to properly process VSV-G protein. Thus, although as visualized by immunofluorescence transport from the ER to the Golgi area was enhanced during IBMX + FK treatment (Fig. 7), the number of VSV-G molecules processed to an endo H-resistant form was decreased in comparison to control cells (Fig. 9A). The same effect was observed when a nondegradable, lipophilic analog of cAMP, Sp-cBIMP’s, was used (not shown). It is important to note, however, that the half-times of processing and the lag periods were similar for both control and IBMX + FK-treated cells (Fig. 9A). Therefore, acquisition of endo H resistance did not provide a reliable indication of ER to Golgi

**Fig. 7.** Visualization of VSV-G exocytic transport in cells treated with IBMX + FK. Infected cells were processed as indicated in Fig. 3 except that they were incubated or not (control) at 32 °C with 500 μM IBMX + 250 μM FK for the indicated time points before fixation and immunofluorescence staining with anti-VSV-G antibody. Cell surface staining is indicated with arrowheads and Golgi staining by arrows. Bar, 25 μm.

**Fig. 8.** Effects of an increased level of cAMP on Golgi structural organization. Noninfected cells were incubated at 37 °C with either 500 μM IBMX + 250 μM FK for 1 h (A and C), 1 μg/ml cholerae toxin for 12 h (D–H), or alternatively, in control medium with no addition (B). Fixed cells in A and D–H were processed for conventional electron microscopy, whereas those in B–C were used for immunogold labeling. A, ultrastructure of the Golgi region after treatment with IBMX + FK; c, centriole; GS, altered Golgi stack. B–C, cryosections were immunolabeled with antibodies against TGN38 (small gold particles, arrows) and α-1,2-mannosidase II (large gold particles, arrowheads). D–H, consecutive serial sections through a Golgi fused compartment. A single mitochondrion is indicated (asterisk). Bars, 0.25 μm.
transport from the TGN to the cell surface was shown to be dependent on activated protein kinase A (Figs. 3 and 4), whereas transport from the ER to the cis-middle Golgi (Fig. 5) or from the intermediate compartment to the TGN (Fig. 6) was sensitive to but did not require protein kinase A activation. This could reflect the involvement of particular transport molecules in each case. In a similar way, the effects of protein kinase A activation on transport activities can differ from one cell system to another. In Madin-Darbin canine kidney-polarized cells, for example, transport from the ER to the Golgi and from the TGN to the basolateral surface was insensitive to protein kinase A activation, whereas transport to the apical surface was stimulated (7). Therefore, it is possible that the protein kinase A substrates relevant to transport processes would be selective proteins required for a particular transfer event rather than molecules involved in multiple transport reactions.

Transport from the TGN to the cell surface was blocked by H-89 (Fig. 4B) and stimulated by IBMX + FK treatment (Fig. 9B). The first agent has been shown to specifically inhibit protein kinase A (30), whereas it is commonly accepted that incubation with either IBMX or FK leads to protein kinase A activation by increasing the intracellular level of cAMP. In principle, the increased number of VSV-G molecules detected at the surface of cells chased in the presence of IBMX + FK could result from an inhibitory effect of these agents on protein uptake by endocytosis. However, we used [125I]-labeled epidermal growth as a ligand for receptor-mediated endocytosis, and we determined that the internalization rates did not differ significantly between control and treated cells. It is then plausible to assume that an increased level of cAMP caused by IBMX + FK incubation stimulate export of VSV-G from the TGN to the cell surface. Similarly, the inhibitory effect of H-89 cannot be explained by a decrease in the cellular levels of ATP necessary for transport (Fig. 1) (36). Instead, the data support a positive regulatory role for protein kinase A in vesicle transport from the TGN to the cell surface in nonpolarized cells. This transport step has been commonly considered to occur constitutively. However, recent reports indicate that it is influenced by different signal transduction molecules (6, 12). In this context, changes in the intracellular concentration of cAMP and other second messengers could control vesicle formation and/or protein sorting at the TGN by exerting a regulatory role on the protein machinery responsible for these activities.

In general results obtained with IBMX and FK were consistent with a stimulatory role of activated protein kinase A on transport. However, these agents also gave rise to important modifications of Golgi structure which could mask their influence on transport activities. Thus, although by immunofluorescence ER to Golgi transport was accelerated in IBMX + FK-treated cells (Fig. 7), acquisition of endo H resistance was hindered in the altered Golgi complex (Fig. 9A). Also, transport out of the Golgi complex was decreased in cells exposed to IBMX and FK for a prolonged time causing retention of VSV-G protein in this organelle (Fig. 7H). For these reasons we believe that both the ability to process glycoproteins and to produce functional transport vesicles were affected in the Golgi complex modified by IBMX and FK treatment. The modifications consisted in intercisterna fusion within the Golgi stacks which gave rise to irregular Golgi compartments and miscompartmentalization of resident Golgi proteins (Fig. 8, A and C). These structural changes were derived from an increase in the intracellular level of cAMP since they were also observed in

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**DISCUSSION**

The transport machinery responsible for vesicle budding, targeting, and fusion is under regulation. Protein kinases and second messengers seem to be involved in this control. We have studied the role played by cAMP and protein kinase A on protein transport along the exocytic pathway. The results indicate that protein kinase A stimulates several transport steps in this route although the extent of regulation varies. Thus, 3 M. Alonso, M. Muñiz, J. Hidalgo, and A. Velasco, unpublished observations.
cells incubated with cholerae toxin (Fig. 8, D–H) or treated with cAMP analogs. Interestingly, an increase in cAMP has been reported to induce alkalization of the trans-Golgi lumen (37) and to affect protein glycosylation (6, 38). In any case, these observations suggest a regulatory role for protein kinase A on the maintenance of the structural organization of the Golgi complex.

Protein kinase A is an ubiquitous regulatory enzyme activated by cAMP binding which in turn phosphorylates specific protein substrates. Cell signaling processes that are mediated by protein kinase A begin with the activation of a plasma membrane receptor coupled to heterotrimic G proteins. These modulate cAMP production by acting on cAMP-phosphodiesterase and adenylyl cyclase. It is becoming increasingly evident that a similar signaling pathway controls intracellular membrane trafficking. Several kinds of heterotrimeric G proteins, including Gaα, Goα, and XLaα proteins, have been detected in intracellular membranes and implicated in vesicular transport along both the exocyctic and endocyctic routes (1, 39–43). It is postulated that these proteins transmit signals to effectors that could regulate transport in a still undetermined way. While ER- and Golgi-associated protein kinases (17, 19) are potential downstream effectors, other evidences suggest that second messengers and protein kinases are not necessarily involved (44). Therefore, it will be important to determine in which way protein kinase A controls transport reactions.

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