Protein Kinase C μ Is Located at the Golgi Compartment

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Abstract. Protein kinase C μ (PKCμ) displays unusual structural features like a pleckstrin homology domain and an amino-terminal hydrophobic region with a putative leader peptide and transmembrane sequence. As a discrete location often is a direct clue to the potential biological function of a kinase, antibodies directed against unique amino- and carboxy-terminal domains of PKCμ were used to localize the protein within intracellular compartments in immunofluorescence and subcellular fractionation studies. Confocal laser scanning microscopy showed colocalization of PKCμ with the resident Golgi marker protein β1,4 galactosyltransferase in PKCμ transfectants and in the human hepatocellular carcinoma cell line HepG2, expressing endogenous PKCμ. Long-term treatment of cells with brefeldin A, which disintegrates the Golgi apparatus, disrupted PKCμ-specific staining. Cosegregation of PKCμ with β1,4 galactosyltransferase, but not with the endosomal marker rab5, upon density gradient fractionation and Western blot analysis of HepG2 cell extracts, provides independent evidence for a Golgi localization of PKCμ. Moreover, cellular sulfate uptake and Golgi-specific glycosaminoglycan sulfation was enhanced in PKCμ transfectants. Together, these data suggest that PKCμ is a resident protein kinase of the core Golgi compartment and is involved in basal transport processes.

THE protein kinases C (PKC)[1] define a family of serine/threonine-specific kinases that are considered as important regulatory enzymes involved in multiple cellular responses. They are activated by lipid second messengers, predominantly diacylglycerol (Nishizuka, 1992), in response to various extracellular agonists like hormones, neurotransmitters, growth factors, and cytokines (Hug and Sarre, 1993; Dekker and Parker, 1994). So far, 11 PKC isoforms have been characterized at the molecular level. Based on the primary structure and in vitro activation requirements, the PKC family can be grouped into three major classes: Ca^{2+}-dependent PKCs (cPKCα, β1, β2, and γ) (Knopf et al., 1986), Ca^{2+}-independent, novel PKCs (nPKCε, δ, η, and θ) (Ohno et al., 1988; Ono et al., 1988; Osada et al., 1990, 1992), and atypical PKCs (aPKCζ and ι/λ; Ono et al., 1989; Selbie et al., 1993; Akimoto et al., 1994). The recently identified novel member PKCμ and its mouse homologue protein kinase D (PKD) do not conform to either one of these major classes and thus may comprise a new subgroup (Johannes et al., 1994; Valverde et al., 1994; Dekker and Parker, 1994). The lack of a typical pseudosubstrate site as well as the presence of two unique amino-terminal hydrophobic domains, together with the unusually large size of the molecule, are characteristic features of PKCμ/PKD. Furthermore, the presence of a pleckstrin homology (PH) domain (Gibson et al., 1994) in the regulatory region of PKCμ/PKD is so far unique within the PKC family. The novel structural features of the isozyme are corroborated by analysis of in vitro kinase activity of PKCμ, revealing a distinct sensitivity profile to various kinase inhibitors with strong inhibition of constitutive kinase activity by the typical protein kinase A inhibitor H89 (Johannes et al., 1995; Dieterich et al., 1996). Moreover, expression and functional analysis of PKD showed an atypical substrate specificity in vitro (Valverde et al., 1994; Van Lint et al., 1995). At present, the understanding of the physiological role of the various PKC members including PKCμ is still limited. Nevertheless, the available structural and biochemical data suggest that PKCμ is likely to have a cellular function that differs from other PKC subtypes.

As complex processes of cellular metabolism require a high degree of functional organization, which is achieved by compartmentalization into organelles, a first step toward understanding the biological function of novel proteins is made by an analysis of tissue-specific expression and subcellular location. For several PKC isozymes, an experimental model of dynamic compartmentalization indicates that, upon binding of physiological activators, enzyme activation leads to the translocation of cytosolic

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1. *Abbreviations used in this paper*: GAG, glycosaminoglycan; PH, pleckstrin homology; PIP_2, phosphatidylinositol-4,5-biphosphate; PKC, protein kinase C; PKD, protein kinase D.

PKCs to the particulate fraction such as plasma membrane, nucleus, or cytoskeletal structures (Hug and Sarre, 1993). Other PKC isozymes are not translocated upon activation, but they appear to be constitutively associated with distinct organelles (Goodnight et al., 1995; Germano et al., 1994; Chida et al., 1994), although, for the same isozyme, different locations have been described for different cell types (Germano et al., 1994; Goodnight et al., 1995). Despite potentially selective intracellular locations, as yet there is no definitive proof of a given isotype being activated in a specific location that would lead to the phosphorylation of a selective substrate. Nevertheless, tissue-specific expression and differential intracellular location suggest distinct functions of individual PKC isotypes in signal transduction and cellular metabolism.

To obtain insights into the potential biological role of PKCμ, we have used PKCμ-specific antibodies and antisera to localize PKCμ within subcellular compartments of stable transfectants and cell lines expressing endogenous PKCμ. Using indirect immunofluorescence and confocal laser scanning microscopy, as well as subcellular biochemical fractionation, we demonstrate that PKCμ is located at the Golgi complex. Furthermore, sulfate uptake as well as Golgi-specific sulfation and release of glycosaminoglycan (GAG) chains were found to be enhanced in PKCμ transfectants, indicating an involvement of PKCμ in constitutive transport processes at the Golgi apparatus.

Materials and Methods

Cell Culture and Transfection

HeLa, CHO, and NIH3T3 (all obtained from American Type Culture Collection, Rockville, MD) were transfected with PKCμ within the pBMGneo vector as previously described (Johannes et al., 1994). In the case of CHO and NIH3T3 cells, neomycin selection was carried out in the presence of 1 mg/ml G418-sulfate (GIBCO BRL, Gaithersburg, MD) for 15 d before picking individual clones. PKCμ expression of transfectants was verified by immunoprecipitation at the time of the experiments.

Immunoprecipitation and Autophosphorylation

The production of an mAb JP1 directed against an amino-terminal PKCμ/glutathione-*S*-transferase fusion protein has been described previously (Johannes et al., 1994). It was used for immunoprecipitation and immunofluorescence studies (see below). In addition, a polyclonal rabbit antiserum, raised against a synthetic peptide corresponding to the carboxy-terminal 18 amino acids of PKCμ (generously provided by D. Fabbro, Ciba Geigy, Switzerland), was used for immunodetection. For immunoprecipitation of PKCμ from the cell line HepG2, cells were lysed by sonification and subsequent incubation in lysis buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 150 mM NaCl, 10 μg/ml leupeptin, 3 μg/ml trasylol, 1 mM PMSF) for 30 min at 0°C. After centrifugation of cell debris, immunoprecipitation was performed with anti-PKCμ mAb (3 μg per 10^7 cells) for 4 h at 4°C. Immunocomplexes were isolated by incubation with protein G–Sepharose for 30 min at 4°C, washed in lysis buffer, and resuspended in 40 μl phosphorylation buffer (20 mM Tris, pH 7.4, 5 mM magnesium acetate, 1 mM DTT). The reaction was started by adding 0.3 μl [γ-^{32}P]ATP (5,000 Ci/mmol, 10 mCi/ml; Amersham Buchler GmbH, Braunschweig, Germany) and incubated for 10 min at 37°C. Reactions were terminated by adding an equal volume of sample buffer, resolved by SDS-PAGE under reducing conditions, dried, or transferred to nitrocellulose membrane, and ^{32}P-labeled proteins were visualized by autoradiography. Western blot analyses of COS PKCμ and of HeLa PKCμ-expressing transfectants (Johannes et al., 1995), as well as of the parental cell lines (equivalents of 0.5×10^6 cells analyzed in each lane) and of HepG2 immunoprecipitates, were performed with anti-PKCμ mAb (0.5 μg/ml) or rabbit immune serum at a 1:250 dilution using an enhanced chemiluminescence detection system (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Immunofluorescence Staining and Confocal Laser Scanning Microscopy

Cells were grown on coverslips to 70% confluency, washed with PBS, and fixed in 3.5% paraformaldehyde in PBS for 15 min at room temperature. Permeabilization of the cells proceeded through incubation with 0.5% saponin, 0.2% BSA, 2% FCS, and 5 mM $MgCl_2$ in PBS for 4 min. All further washes and incubations were carried out in the same solution containing 0.05% saponin. For detection of β1,4 galactosyltransferase and/or PKCμ, cells were incubated for 2 h with an anti-β1,4 galactosyltransferase antiserum (obtained from E. Berger, University of Zürich, Switzerland) diluted 1:50 and/or with an anti-PKCμ mAb (0.3 μg/μl), followed by incubation with goat anti–rabbit IgG–FITC conjugate and/or goat anti–mouse IgG–Cy3 conjugate (Sigma Chemical GmbH, Munich, Germany) for 1 h. The coverslips were mounted in Moviol mountant supplemented with 0.1% *p*-phenylendiamine and photographed with a fluorescence microscope (Axiovert; Zeiss, Oberkochen, Germany). Confocal laser scanning images were taken by a Fluovert microscope using a TCS4D scanner (Leica Instruments GmbH, Nussloch, Germany). Excitation intensities of both fluorescent dyes were cross-corrected, and areas of colocalization were represented in blue. The optical slice in z-direction corresponds to 0.5 μm. Brefeldin A incubations were performed at a final concentration of 2 μg/ml for 30 min at 37°C. PMA stimulation of HepG2 cells was performed at 100 ng/ml for 20 min.

Subcellular Fractionation

Isolation of Golgi membrane–enriched subcellular fractions by velocity-controlled sucrose gradient fractionation was performed as described (Balch et al., 1984). In brief, 4×10^7 HepG2 cells were resuspended in 1.75 ml 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, and homogenized by applying 20 strokes with a "very tight-fitting" 5-ml Dounce homogenizator (Braun, Melsungen, Germany). The cellular extract was adjusted to 1.4 M sucrose by adding 1.75 ml 2.3 M sucrose and overlaid upon two layers of 0.6 ml 2 M sucrose and 1.2 ml 1.6 M sucrose. The cellular extract was overlaid with 3.5 ml 1.2 M sucrose and 2.3 ml 0.8 M sucrose and centrifuged in a rotor (SW28; Beckman Instruments, Inc., Palo Alto, CA) for 2.5 h at 25,000 rpm. Three distinct interphases, as shown schematically in Fig. 4, were obtained. Equal amounts of protein (10 μg for β1,4 galactosyltransferase and 30 μg for PKCμ and rab5 detection) in each lane were applied to a 10 or 12% SDS-PAGE, followed by Western blot analysis using either an anti-PKCμ (Johannes et al., 1995) or an anti–β1,4 galactosyltransferase (Nilsson et al., 1993) antiserum in a 1:1,000 dilution using an alkaline phosphatase–based detection system. The rab5-specific antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), serving as a marker for the early endosome, was used in a dilution 1:250.

Quantitative evaluation of Western blots was performed by densitometric scanning using a video imaging system with Bioprofil Software (Froebel, Lindau, Germany). Arbitrary units of PKCμ and β1,4 galactosyltransferase were estimated by scanning the alkaline phosphatase–stained blots. The Western blot signal obtained from 10 μg lysate for β1,4 galactosyltransferase, and 30 μg lysate in the case of PKCμ, was set at one, and relative amounts of fractionated antigen were calculated by multiplying arbitrary units with the total protein content of each fraction.

Sulfation of Glycosaminoglycans

As an indicator of Golgi-specific function, GAG was measured by a modification of the GAG release assay described by Miller and Moore (Miller and Moore, 1991; Lehel et al., 1995) for a 6- or 24-well plate. Transfectants were grown to confluence (5×10^5 cells for the 6-well plate and 3×10^5 for the 24-well plate) and serum starved overnight. Expression of PKCμ was induced by adding 1 μM $CdCl_2$ for 4 h. Serum-deprived cells were induced 30 min with the xylose analogue 4-methylumbelliferyl β-D-xyloside (designated xyloside) at a final concentration of 1 mM in buffer A (20 mM Hepes, pH 7.2, 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 g/liter glucose). The cells were labeled with $Na_2[^{35}S]SO_4$ (Amersham Buchler GmbH; 17 μCi per well in 300 μl of buffer A for the kinetics, and 5.5 μCi per well in 150 μl for all other experiments) for 10 min, rapidly washed with 1 ml DMEM containing 4 mM unlabeled Na_2SO_4, and subsequently incubated in 1 ml DMEM to allow GAG secretion. To measure the kinetics of GAG chain secretion, 50-μl

aliquots of the culture medium were removed at the indicated time points, and 150 μl of buffer A, chondroitinsulfate (5 μl of a 10 mg/ml stock solution), and cetylpyridinium chloride (5 μl from a 10% wt/vol solution) was added. GAG chains were precipitated at 37°C for 1 h. Precipitates were collected on a nitrocellulose-covered microtiter plate (Pall Biosupport, East Hills, NY) and washed with 0.8 ml 1% cetylpyridinium chloride and 40 mM Na_2SO_4 per well. The filters were dried and counted in 2 ml scintillation fluid. At the end of the experiment, the cells were lysed in 0.5 ml of buffer B (10 mM Hepes, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100), and 200 μl aliquots were subjected to the BCA assay (Pierce Chemical Co., Rockford, IL) to estimate the protein content.

Sulfate Uptake

To measure sulfate uptake into intact cells in the presence of xyloside, 10^5 cells growing on 24-well plates were processed as described for the GAG chain precipitation. Cells were labeled with 5.5 μCi $Na_2[^{35}S]SO_4$ for 10 min. After three washes with 0.4 ml DMEM, cells were harvested in 400 μl buffer B. ^{35}S-incorporation was determined by measuring 100-μl aliquots in 2 ml scintillation fluid. The protein content of each culture was determined using the BCA assay (Pierce Chemical Co.). Nonspecific sulfate uptake was measured by a brief incubation of PKCμ and vector control transfectants with [^{35}S]sulfate on ice followed by three washes with 500 μl DMEM. Nonspecific binding to the cells was <10% of total [^{35}S]sulfate uptake. No significant differences between PKCμ transfectants and vector controls were detected. All assays concerning GAG secretion and sulfate uptake were performed in triplicate. Results shown are representative of at least three similar experiments.

Results

Localization of PKCμ by Indirect Immunofluorescence

To analyze the intracellular location of PKCμ, previously described stable PKCμ-expressing transfectants of COS-1 and HeLa cells (Johannes et al., 1994) were used for immunocytochemical analysis. Immunostaining of the respective transfectants was performed using the previously described mAb JP1 (Johannes et al., 1995). In both COS and HeLa transfectants, the immunofluorescence signals were focused in caplike perinuclear structures, becoming weaker towards the peripheral cytoplasm (Fig. 1, *C* and *G*). Specific staining of the nucleus or the plasma membrane was not observed in either transfectant (Fig. 1, *C* and *G*). No immunoreactive signals could be detected in the nontransfected parental cell lines (Fig. 1, *A* and *E*, vs *C* and *G*, respectively; see also Fig. 2, lanes *2* and *4* vs lanes *1* and *3*). The stained subcellular structures in COS transfectants produced a higher fluorescence intensity as compared with HeLa transfectants, which is in accordance with the higher expression of PKCμ in the former, as revealed by Western blot analysis (see Fig. 2, lane *1* vs lane *3*).

HepG2 Cells Do Express Endogenous PKCμ

Northern blot data from human tissues as well as reverse transcription–PCR from several cell lines (Johannes et al., 1994; data not shown) provided evidence that endogenous PKCμ transcripts are expressed at relatively low abundance. As the above data suggested that PKCμ might function as Golgi-specific PKC, cell lines established from secretory tissues were assayed for PKCμ expression. The human hepatocellular carcinoma celline HepG2 (Aden et al., 1979) was identified as constitutively expressing significant amounts of PKCμ. Immunoprecipitation and subsequent Western blot detection with the mAb JP1, as well as with the polyclonal rabbit antiserum, revealed that HepG2-derived PKCμ migrates as a single band at ~115 kD, in a similar fashion to the recombinant gene product of COS and HeLa cell transfectants (Fig. 2, lane *5* vs lanes *1* and *3*). As expected, HepG2-derived PKCμ showed the same cofactor-independent autophosphorylation activity as recombinant PKCμ expressed in transfectants (Fig. 2, lane *6*; Johannes et al., 1995). PKCμ could be readily detected in $\sim 0.5 \times 10^6$ cell equivalents in transfectants by Western blot analysis and in 2×10^6 equivalents of HepG2 cells by immunoprecipitation.

Localization of PKCμ at the Golgi Apparatus by Confocal Laser Scanning Microscopy

From previously reported biochemical fractionation studies, PKCμ has been localized in the particulate cellular fraction (Johannes et al., 1995). In an attempt to identify more precisely the location of PKCμ in subcellular structures, immunofluorescence double-labeling experiments using antibodies against a variety of cytoskeletal or organelle-specific proteins were performed. No colocalization of PKCμ with actin-containing stress fibers, vimentin-containing intermediate filaments, or Trapα as a marker of the ER (Hartmann et al., 1993) was detected (data not shown).

A conformity in the staining patterns was found in cells labeled in parallel with FITC-coupled WGA that preferentially binds to Golgi structures (Henson et al., 1992; Johnston et al., 1994) and the PKCμ-specific mAb JP1 (data not shown). A similar localization pattern was obtained upon staining with an mAb against the coatomer protein β-COP (Duden et al., 1991; data not shown). β-COP is a main structural component of non–clathrin-coated vesicles, mediating transport between the RER and the *cis*-Golgi (Duden et al., 1991; Pelham, 1994). An overlapping staining pattern of PKCμ with WGA and β-COP suggested a predominant association of PKCμ with the Golgi compartment. To scrutinize this prediction, costaining experiments were performed with a well-established Golgi-specific marker enzyme, β1,4 galactosyltransferase, that has been localized to the medial cisternae of the Golgi (Nilsson et al., 1993). In Fig. 3, a representative colocalization experiment using confocal laser scanning microscopy of PKCμ (*A*, *red fluorescence*) and β1,4 galactosyltransferase (*B*, *green fluorescence*) is shown for HeLa PKCμ transfectants (*upper panels*) and HepG2 cells endogenously expressing PKCμ (*middle* and *lower panels*). A computer-based superimposition by image processing yielded, in each case, an overlay of the β1,4 galactosyltransferase–specific signals with most of the PKCμ-specific signal (Fig. 3, *C*, *F*, *I*, and *L*), indicating staining of the same structures. In addition, in both cell lines, a weaker PKCμ-specific signal was found throughout cytoplasmic structures not stained by the β1,4 galactosyltransferase–specific antibody (Fig. 2, *A* and *D*). Additional hints for a Golgi-localization of PKCμ were obtained by brefeldin A treatment of HepG2 cells. Long-term treatment of cells with brefeldin A, a fungal metabolite, results in complete disintegration of the highly organized Golgi structure (Bauernfeind and Huttner, 1993). As shown in Fig. 3, *G–I*, treatment of HepG2 cells with brefeldin A for 30 min leads to a nearly complete disruption of both the PKCμ- and the β1,4 galactosyltransferase–specific staining. Both signals disappeared

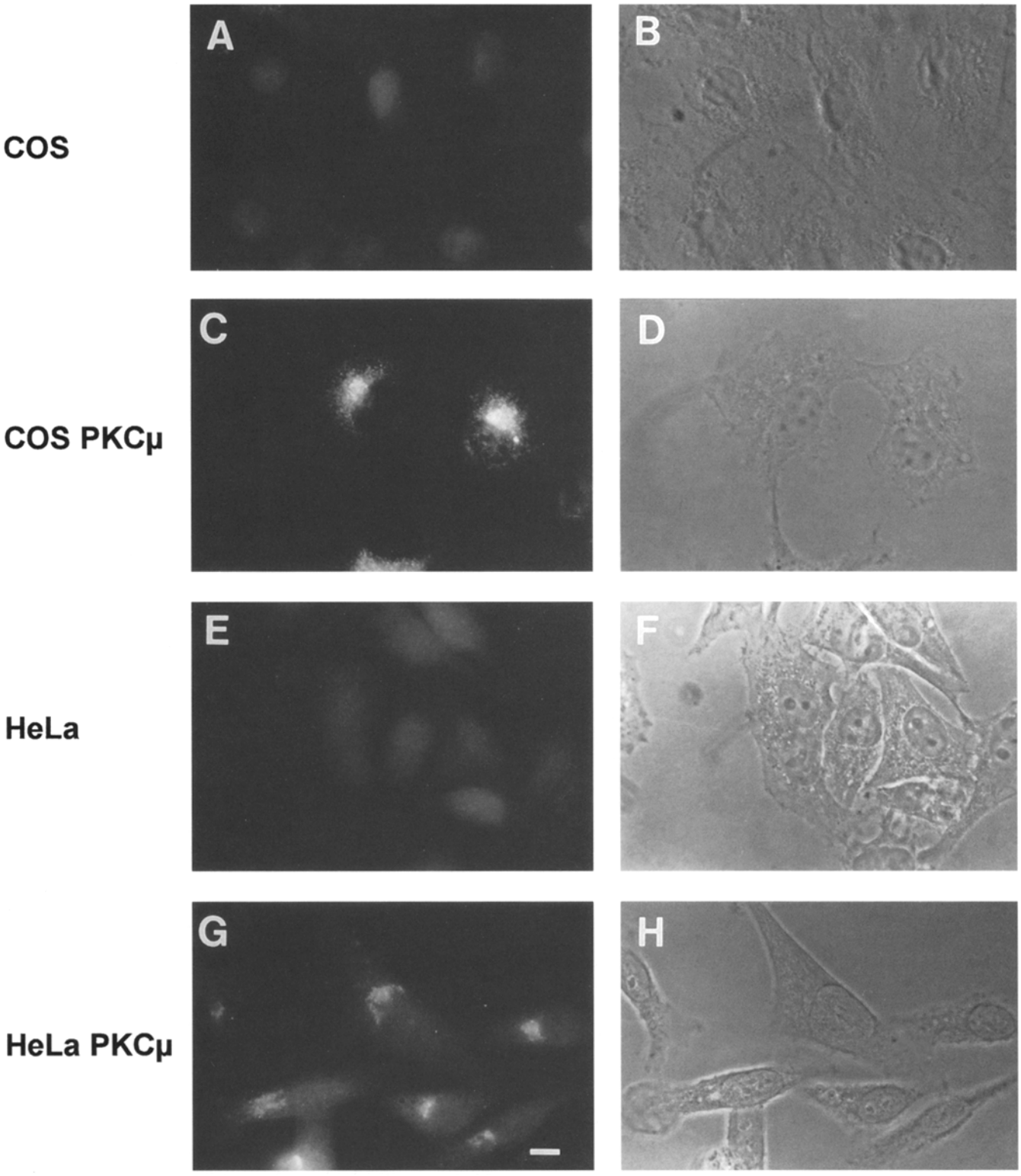


Figure 1. Localization of PKCμ in transfectants by indirect immunofluorescence. COS PKCμ- (*C* and *D*) and HeLa PKCμ- (*G* and *H*) expressing transfectants grown on coverslips were processed for immunofluorescence as described in Materials and Methods. Cells were stained with the PKCμ-specific mAb JP1 and compared with the parental COS (*A* and *B*) or HeLa (*E* and *F*) cell line, respectively. PKCμ was visualized with a Cy3-coupled goat anti–mouse IgG as secondary antibody in *A*, *C*, *E*, and *G*. (*B*, *D*, *F*, and *H*) Respective cells by light microscopy. Bar, 5 μm.

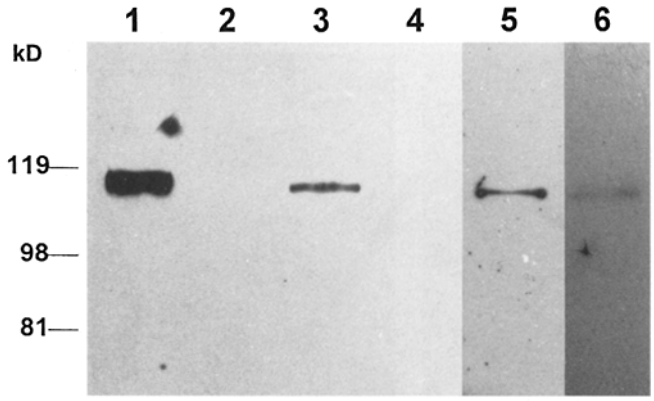


Figure 2. Expression of endogenous PKCμ in the hepatocellular carcinoma cell line HepG2 and recombinant PKCμ in transfectants. PKCμ is detected in extracts from COS and HeLa transfectants and not in the parental cell lines (lanes *1* and *3* vs *2* and *4*, respectively) by Western blot analysis. PKCμ can be immunoprecipitated from HepG2 cells (lane *5*) and autophosphorylated (lane *6*). Rabbit peptide antiserum was used for immuoprecipitation, and the mAb JP1 was used for Western blot detection.

with the same kinetics in brefeldin-treated HepG2 cells (data not shown), indicating that PKCμ and β1,4 galactosyltransferase are localized to the same Golgi subcompartment.

With the exception of the atypical isoforms, activation of PKCs by phorbol esters leads to a rapid redistribution of the enzyme, predominantly from cytosolic to membrane locations. We previously demonstrated that, despite a high affinity phorbol ester binding of recombinant PKCμ in vitro (Dieterich et al., 1996), the phorbol ester binding capacity of extracts from PKCμ transfectants was only slightly increased, and long-term treatment of PKCμ transfectants with phorbol esters did not lead to a proteolytic degradation (Johannes et al., 1994), as is typical for other PKC subtypes (Hug and Sarre, 1993). Next, we investigated whether PMA treatment affects the localization of PKCμ. Upon a 20-min PMA treatment of HepG2 cells, no translocation of PKCμ could be detected (Fig. 3, *J–L*). Interestingly, PMA treatment of HepG2 cells affected the morphology of the Golgi apparatus with an apparent condensation of both PKCμ and β1,4 galactosyltransferase (Fig. 3, *J–L*). This is probably due to considerable changes in the cytoskeletal structures, involving a reorganization of actin filaments upon PMA treatment (Prestle, J., unpublished observations).

Copurification of PKCμ with β1,4 Galactosyltransferase in Fractionated Cell Extracts

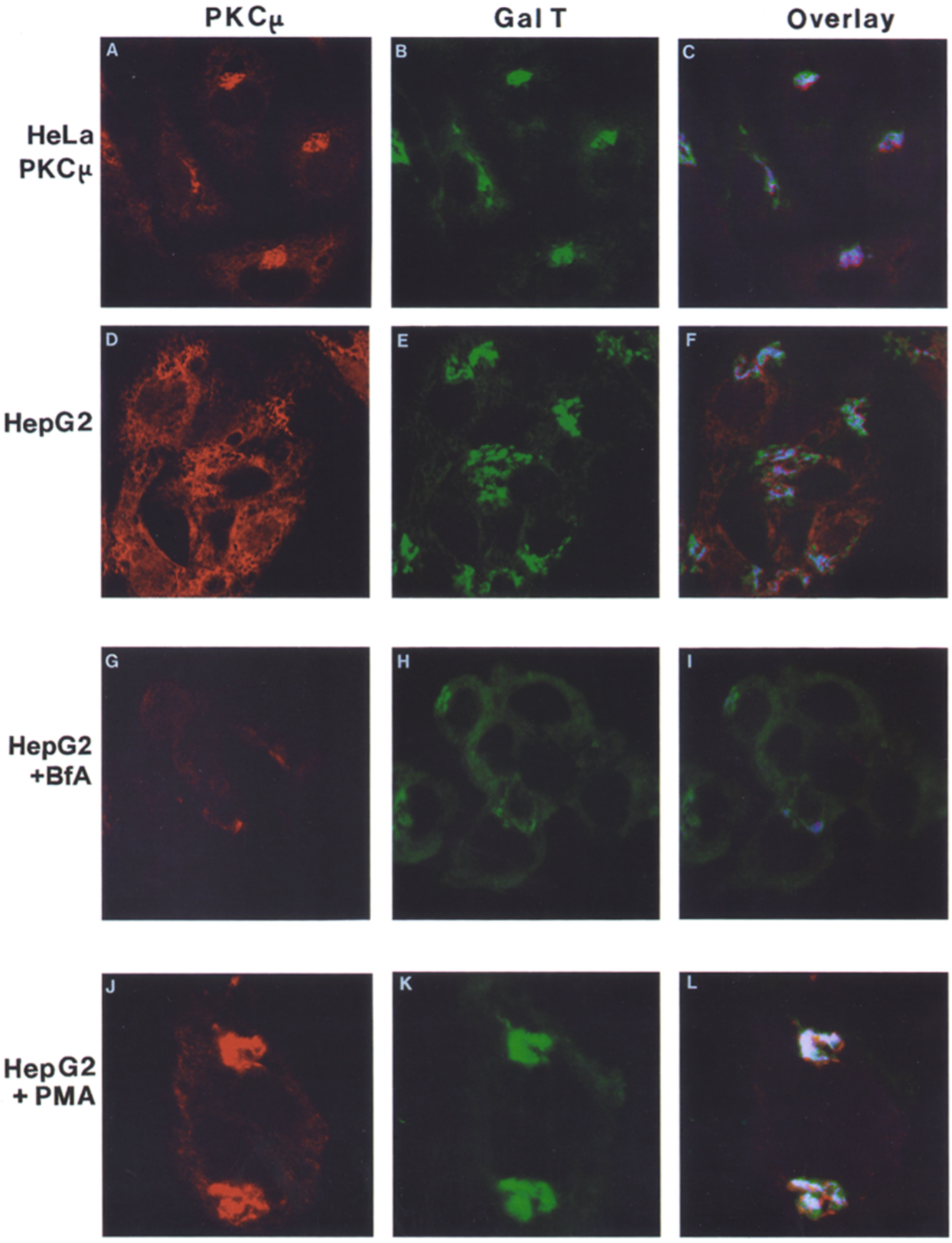
The predicted Golgi association of PKCμ was investigated in an independent biochemical approach by Western blot analyses of isolated Golgi membranes. From HepG2 cells expressing endogenous PKCμ (Fig. 2), Golgi membrane–enriched subcellular fractions were isolated by differential cell fractionation on sucrose cushions (Balch et al., 1984). As judged upon SDS-PAGE and Western blot analysis of fractions from sucrose layers, most of the β1,4 galactosyltransferase and PKCμ immune reactivity was found in the interphase between the 1.4 and 1.6 M sucrose cushions (Fig. 4, fraction *6*). Both PKCμ and Golgi membrane–bound β1,4 galactosyltransferase were recovered in the same fractions using a rabbit serum directed against a carboxy-terminal epitope of PKCμ and an antiserum against β1,4 galactosyltransferase, showing copurification of both proteins (Fig. 4). Calculation of the relative distribution of PKCμ showed similar distribution with ~41% of total β1,4 galactosyltransferase recovered in interphase I (fraction *6*), in which peak activity (55%) of PKCμ was also detected (see Table I).

The Golgi membrane–enriched fractions from endogenous PKCμ-expressing HepG2 cells were further analyzed for the presence of a marker protein representative of other subcellular compartments. The distribution of rab5, a member of the rab GTPase family (Pfeffer, 1994), which acts as a regulatory factor in the early endocytic pathway and is considered a marker of the early endosome (Bucci et al., 1992), was used as an indicator to verify appropriate separation of the distinct organelle membranes during the fractionation procedure. As shown by Western blot analysis of the sucrose gradient fractions (Fig. 4, *lower panel*), the peak activity of rab5 was found in fraction 2 (representing interphase III), which is clearly separated from the peak of β1,4 galactosyltransferase and PKCμ (Fig. 4, fraction *6*).

HeLa PKCμ Transfectants Show an Enhanced Sulfate Uptake and Constitutive Secretion of Sulfated Glycosaminoglycans

Previous studies have established that the Golgi apparatus is the exclusive site for sulfation reactions (Vertel et al., 1993). The measurement of incorporated ^{35}S-labeled sulfate into glycosaminoglycans can be used to follow constitutive protein secretion in cells (Miller and Moore, 1991; Chanat and Huttner, 1991). To determine whether Golgi-associated PKCμ might modulate Golgi function, the kinetics of GAG release was measured in HeLa PKCμ transfectants and compared with mock-transfected HeLa cells. As shown in Fig. 5 *A*, PKCμ transfectants displayed an enhanced sulfation rate, as evident from 89% of GAG secretion of HeLa transfectants compared with 60% of vector controls. The enhanced glycosaminoglycan release could be confirmed in several independent HeLa PKCμ cell clones (Fig. 5 *B*). In addition to the analyzed HeLa transfectants, PKCμ-expressing cell lines originating from different tissues and species were established. In CHO and NIH3T3 transfectants, PKCμ protein expression correlated with enhanced GAG chain secretion. As shown in Fig. 5 *B*, all PKCμ transfectants displayed an enhanced secretion capacity compared with the parental cell lines and/or mock-transfected control clones.

Sulfation of proteins is a multistep process in which the rate-limiting reactions can occur at different steps. An early rate-limiting step is the transport of sulfate into cells and further on to the Golgi compartment. Therefore, HeLa PKCμ transfectants were analyzed for differences in sulfate uptake. As shown in Fig. 6, both in the absence or presence of xyloside, HeLa PKCμ transfectants display an enhanced sulfate uptake compared with control transfectants or parental cells. Together, these data indicate an in-


PKCμ
Gal T
Overlay
HeLa PKCμ
HepG2
HepG2 +BfA
HepG2 + PMA
A
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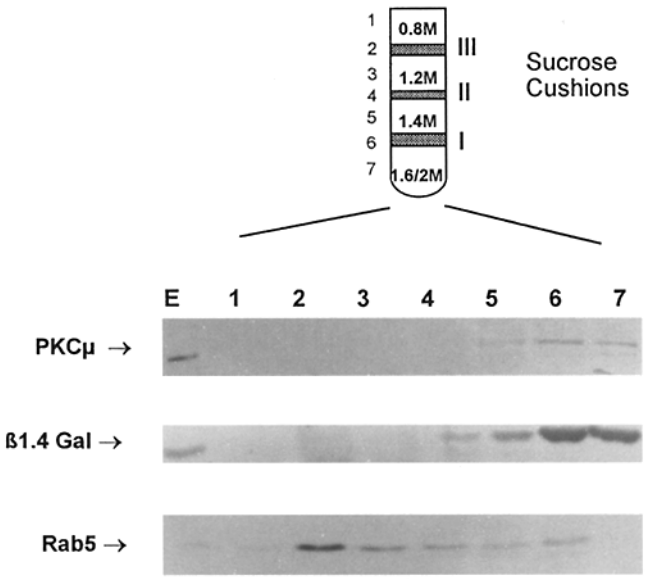

Figure 4. Copurification of PKCμ with β1,4 galactosyltransferase in Golgi membrane–enriched subcellular fractions. 40 × 10^6 HepG2 cells were homogenized and fractionated on sucrose cushions as indicated at the top of the figure. TCA-precipitated fractions were subjected to SDS-PAGE, followed by Western blot analyses with a PKCμ-, β1,4 galactosyltransferase–, and rab5-specific polyclonal rabbit antiserum as described in the Materials and Methods section. Equal loading of each lane was verified by Ponceau staining of the nitrocellulose membrane before Western blot analysis. *E*, cellular extract.

volvement of PKCμ in generation and/or secretion of sulfated glycosaminoglycans, potentially by regulating, via phosphorylation, the activity of Golgi membrane–located transporters.

Discussion

Protein phosphorylation is a fundamental process in regulating cellular transport processes (Davidson et al., 1992), and different lines of evidence have previously pointed out a role of PKCs in vesicular transport (Coffey et al., 1993). A requirement for PKC has been demonstrated in regulated exocytosis (Akita et al., 1994) and for the movement of cell surface receptors through the endocytic pathway (Cardone et al., 1994). Moreover, a calphostin C–sensitive protein kinase C, insensitive to downregulation by long-term phorbol ester treatment, has been postulated to be involved in the export of vesicular stomatitis virus glycoprotein from the ER to the *cis*-Golgi network (Fabbri et al., 1994). In other models, an undefined PKC subtype has been shown to play a critical role in the regulation of the assembly of GTP-dependent binding of the coat protein ADP-ribosylation factor and β-COP to Golgi membranes in rat basophil leukemia cells (De Matteis et al., 1994). In the present study, we provide evidence for a constitutive location and functional role of PKCμ at the core Golgi compartment of HepG2 cells and various transfectants by three distinct experimental approaches. First, indirect immunofluorescence staining of PKCμ revealed an exclusive caplike staining pattern in a perinuclear region. Further analyses by confocal laser scanning microscopy, using the Golgi marker β1,4 galactosyltransferase (Nilsson et al., 1993) and a PKCμ-specific antibody, showed that both signals could be superimposed. Second, independent evidence for a Golgi association was obtained from cell fractionation studies on sucrose cushions (Balch et al., 1984) and subsequent marker colocalization by Western blot analysis. Using this protocol for enrichment of Golgi membranes, PKCμ precisely comigrated in fractions containing the Golgi-specific marker protein β1,4 galactosyltransferase and segregated from the early endosomal marker rab5. Third, Golgi localization and selective function of PKCμ is corroborated by specific enhancement of cellular sulfate uptake and of sulfated GAG chain release in PKCμ transfectants of different origin, further indicating that PKCμ is a resident Golgi protein in these cell lines. Therefore, we propose that PKCμ could be involved in the regulation of Golgi transport processes.

Another PKC subtype, PKCε, has also been reported to be localized at the Golgi compartment (Lehel et al., 1995). Interestingly, both isozymes appear to be involved in regulation of secretion; PKCμ and PKCε modulate Golgi-specific glycosaminoglycan sulfation in cell culture, yet in opposite directions: PKCμ enhances (Fig. 5) and PKCε

Table I. Cofractionation of β1,4 Galactosyltransferse and PKCμ in Purified Golgi Membranes by Sucrose Gradient Centrifugation

Fraction	PKCμ		β1,4 Galactosyltransferase	
	U	%	*U*	%
1	0.2	0.3	0.4	0.1
2	0.7	0.8	0.9	0.3
3	0.5	0.5	2.9	0.9
4	1.9	1.7	42.9	12.9
5	15.1	13.7	43.1	12.9
6	61.5	55	137	41.1
7	31.1	28	105.8	31.7
Cell Extract	111	100	333	100

Arbitary units of PKCμ and β1,4 galactosyltransferase were estimated by scanning Western blots shown in Fig. 4. One arbitrary unit is defined as the signal detected with the PKCμ antiserum and with the β1,4 galactosyltransferase antiserum in 30 and 10 μg total cellular extract, respectively.

Figure 3. Colocalization of PKCμ with Golgi-specific marker proteins using confocal laser scanning microscopy. Hela PKCμ transfectants (*A–C*) and HepG2 cells (*D–L*) were grown on coverslips and processed as described in Materials and Methods. (*A*, *D*, *G*, and *J*) PKCμ-specific staining using the mAb JP1. (*B*, *E*, *H*, and *K*) β1,4 Galactosyltransferase–specific staining using a polyclonal antiserum. (*C*) Superimposition of *A* and *B* after cross-correction of both fluorescence signals. Areas of colocalization (*blue*). (*F*) Combination of *D* and *E*, showing a colocalization of β1,4 galactosyltransferase (*GalT*) and PKCμ in HepG2 cells. (*G–I*) Same staining and overlay as in *D–F* for HepG2 cells after incubation for 30 min with brefeldin A (2 μg/ml). (*J* and *K*) HepG2 cells after stimulation for 20 min with PMA (100 ng/ml).

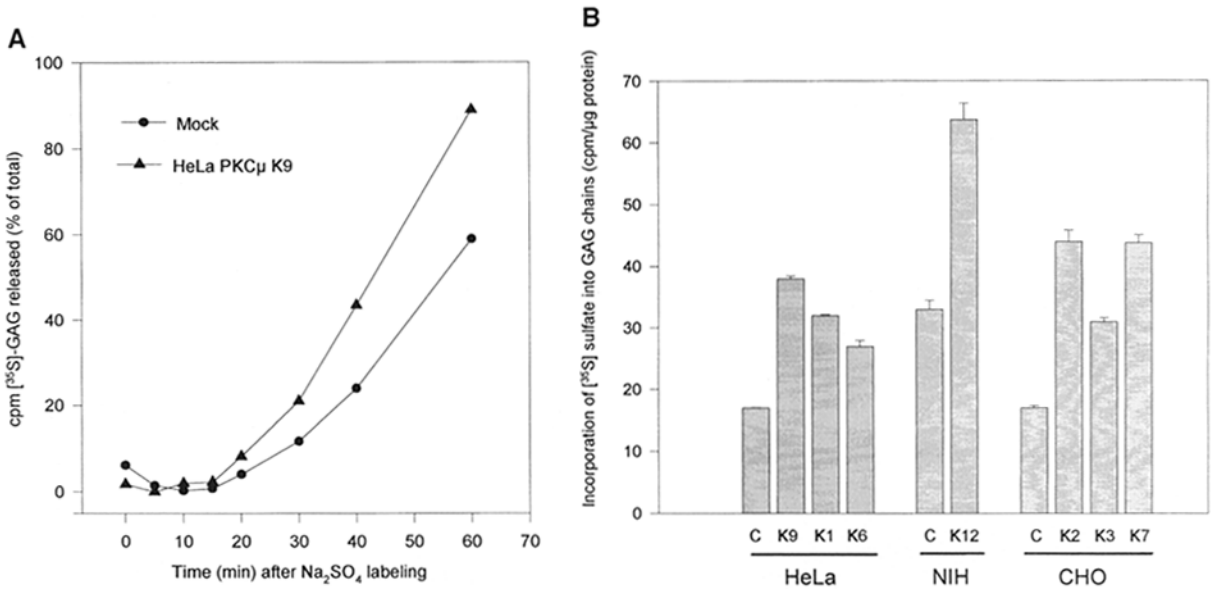

Figure 5. Enhanced secretion of sulfated glycosaminoglycans in PKCμ transfectants. (*A*) Kinetics of GAG release in a HeLa PKCμ transfectant compared with a vector control. Cells grown on 6-well plates were incubated overnight in serum-free DMEM. PKCμ expression was induced by incubation with 1 μg/ml $CdCl_2$ for 4 h. Cells were starved for 30 min in sulfate-free buffer A containing 0.5 mM xyloside, and then labeled for 10 min at 37°C with $[^{35}S]SO^4$. The cells were washed to remove excess of $[^{35}S]SO^4$ and chased for 60 min in DMEM. At the indicated times, aliquots were removed, and secreted GAG chains were precipitated as described in the Materials and Methods section. (*B*) Enhanced release of GAG chains in different cell types. The ^{35}S-labeled GAG chains present in the medium and cell lysates were quantitated and added together. 3×10^5 cells were grown in 24-well plates and processed as described above. After a 30-min chase, GAG chains were precipitated from the cells and the supernatant in a filter assay, quantitated by liquid scintillation counting, and added together. Cells were processed as described for *A*. Three independent clones, in the case of HeLa transfectants (K9, K1 and K6), and CHO transfectants (K2, K3 and K7), and one clone (K12), in the case of NIH transfectants, were analyzed. (*C*) Controls were either vector transfectants (*HeLa* and *NIH* cells) or the parental cell line (*CHO* cells). Error bars indicate the range of triplicate determinations.

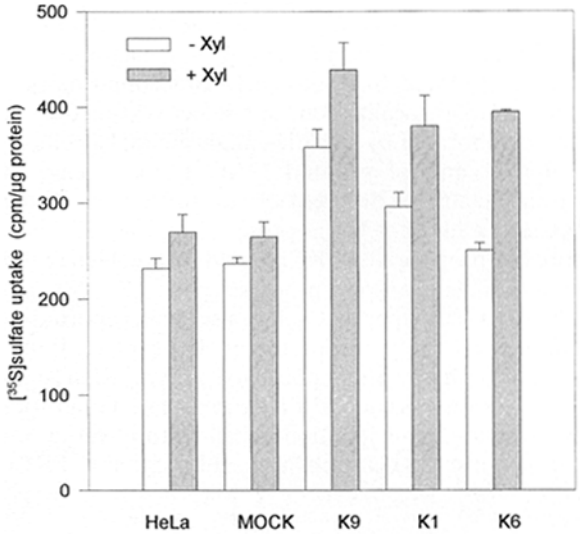

Figure 6. Sulfate uptake is enhanced in HeLa PKCμ transfectants. 10^5 cells were grown in 24-well plates and processed as described in the legend of Fig. 5. After labeling, the cells were washed three times with 500 μl DMEM to remove free $[^{35}S]SO_4$. The cells were lysed, and incorporated $[^{35}S]SO_4$ was measured by liquid scintillation counting. Error bars indicate the range of triplicate assays.

inhibits (Lehel et al., 1995) sulfated GAG chain release, respectively. Accordingly, as GAG chain release is a paradigm of constitutive secretion (Chanat and Huttner, 1991), these data make PKCμ and PKCε good candidates for antagonistic regulators of secretory processes.

Of particular significance for regulation of PKCμ activity at the Golgi could be the finding that PKCμ possesses a PH-domain in its regulatory region (Gibson et al., 1994). PH-domains are structural elements of various proteins known or supposed to play a role in signal transduction and, in analogy to SH2 and SH3 domains, are currently considered as potential modules for protein–protein interactions. Although to date limited data about direct interaction of defined proteins via the PH-domain exist (Pawson, 1995), there is evidence that certain PH-domains contribute to the binding of the βγ subunit of heterotrimeric G-proteins (Koch et al., 1993). Novel and classical heterotrimeric G-proteins appear to play critical roles in different steps of vesicular transport through the Golgi apparatus (Kehlenbach et al., 1994; Stow et al., 1991; Bauerfeind and Huttner, 1993) and could potentially interact with the PKCμ PH-domain.

Independent of a potential function as a protein-interaction domain, PH-domains have been described as target structures of phospholipids. In particular, specific association with phosphoinositides, e.g., phosphatidylinositol-4,5-bisphosphate (PIP_2), has been demonstrated for several PH-domains (Harlan et al., 1994). In the context discussed here, it is noteworthy that a role for phosphoinositides in

regulating vesicular traffic emerges (Burgoyne, 1994). The key regulatory molecule ADP-ribosylation factor has been found to activate phospholipase D in a PIP_2-dependent manner (Kahn et al., 1993). In addition, activity of ADP-ribosylation factor GTPase-activating protein is enhanced by PIP_2 and phosphatidic acid, the product of phosphatidylcholine hydrolysis by phospholipase D (Randazzo and Kahn, 1994). PIP_2 was also shown to be an activator of PKCμ kinase activity (Dieterich et al., 1996). A role of phosphoinositides was already evident from the previous cloning of the vacuolar protein-sorting protein of yeast, VPS34 (Schu et al., 1993). The recent identification of the human homologue of VPS34, a phosphatidylinositol-3-phosphate–specific kinase (Volinia et al., 1995), shows that this sorting mechanism is highly conserved throughout the species.

Based on the above-mentioned evidence for an important role of heterotrimeric G-proteins as well as phosphatidylinositols in Golgi transport processes, and our finding that PKCμ is located at the Golgi, affects sulfate uptake as well as GAG chain release, and possesses a PH-domain, it is possible that PKCμ activity is under control of Golgi-localized G-proteins and/or phosphatidylinositols. With the availability of purified recombinant PKCμ protein, this hypothesis can now be experimentally addressed.

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