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Identification of a high affinity binding protein for the regulatory subunit RIIβ of cAMP-dependent protein kinase in Golgi enriched membranes of human lymphoblasts

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Immunocytochemical evidence of an association between the regulatory subunit RII of the cAMP-dependent protein kinase (cAMP-PK) and the Golgi apparatus in several cell types has been reported. In order to identify endogenous Golgi proteins binding RII, a fraction enriched in Golgi vesicles was isolated from human lymphoblasts. Only the RIIβ isoform was detected in the Golgi-rich fraction, although RIIα has also been found to be present in these cells. A 85 kDa RII-binding protein was identified in Golgi vesicles using a [^{32}P]RII overlay of Western blots. The existence of an endogenous RIIβ–p85 complex in isolated Golgi vesicles was demonstrated by two independent means: (i) co-immunoprecipitation of both proteins under non-denaturing conditions with an antibody against RIIβ and (ii) co-purification of RIIβ–p85 complexes on a cAMP-analogue affinity column. p85 was phosphorylated by both endogenous and purified catalytic subunits of cAMP-pKII. Extraction experiments and protease protection experiments indicated that p85 is an integral membrane protein although it partitioned atypically during Triton X-114 phase separation. We propose that p85 anchors RIIβ to the Golgi apparatus of human lymphoblasts and thereby defines the Golgi substrate targets most accessible to phosphorylation by C subunit. This mechanism may be relevant to the regulation of processes involving the Golgi apparatus itself, such as membrane traffic and secretion, but also relevant to nearby nuclear events dependent on C subunit.

Key words: cAMP-dependent protein kinase/Golgi apparatus/RIIβ/RII-binding proteins

Introduction

In higher eukaryotes, most of the physiological responses to cAMP are mediated by the activation of the cAMP-dependent protein kinase (cAMP-PK). In the absence of cAMP, cAMP-PK exists as an inactive tetramer containing a regulatory (R) subunit dimer with a catalytic (C) subunit bound to each R subunit. Binding of cAMP to the R subunits results in dissociation of the C subunits, which then become active (reviewed in Taylor *et al.*, 1990). In most mammalian tissues or cells two distinct types of cAMP-PK, named type I and II, have been characterized. The two types of holoenzymes were initially distinguished by properties associated with their R subunits (RI and RII) and cDNA cloning has revealed that additional isoforms of RI (RIα and RIβ, Lee *et al.*, 1983; Clegg *et al.*, 1988) and RII (RIIα and RIIβ, Jahnsen *et al.*, 1986; Scott *et al.*, 1987) exist and that they are encoded by different genes. There is much less amino acid conservation among these R isoforms than among the three C subunit isoforms (α, β and γ, Uhler *et al.*, 1986a,b; Beebe *et al.*, 1990) which are >95% identical (reviewed in Meinecke *et al.*, 1990). Several studies have shown that the different R isoforms have distinct tissue distributions. Whereas the α isoforms of both R subunits appear to be variably expressed in most tissues, the β isoforms seem to be restricted to cerebral and reproductive cells (Erlichman *et al.*, 1980; Jahnsen *et al.*, 1986; Scott *et al.*, 1987; Stein *et al.*, 1987; Clegg *et al.*, 1988; Cadd and McKnight, 1989). R subunits also differ in their intracellular distribution and ability to interact with subcellular structures. Thus, >90% of R subunits in non-neuronal cells are found in the cytosol (Rubin *et al.*, 1979) whereas 50 to 75% of the cerebral cortex RII, predominantly β, is tightly associated to the membrane fraction (Lohmann *et al.*, 1980; Stein *et al.*, 1987).

Kinase anchoring to defined subcellular structures has been shown to be mediated by the regulatory subunit, since cAMP can release C but not RII from the complex (Corbin *et al.*, 1977). RII has been found associated with centrosomes and the Golgi apparatus in both primary cultures of brain cells as well as permanent cell lines such as fibroblast and endothelial cells (Nigg *et al.*, 1985; De Camilli *et al.*, 1986). RII also co-localizes with microtubules in neurons (Theurkauf and Vallee, 1982) and with the flagellum in rat spermatozoa (Horowitz *et al.*, 1988). In canine pancreas, association of RII with fractions containing rough endoplasmic reticulum and the Golgi apparatus has been reported (Nigam and Blobel, 1989). Recently, early endosomes, prelysosomal compartment and the *trans*-Golgi network have been added to the list of subcellular locations containing cAMP-PKII (Griffiths *et al.*, 1990). From these data, a regulatory role for cAMP-PKII in intracellular membrane traffic, secretion, cell polarity, endocytosis and cell surface receptors recycling has been suggested.

Direct interactions between RII and structural components designated RII-binding proteins have been identified. These include three neuronal proteins, the microtubule-associated protein MAP2 (Theurkauf and Vallee, 1982), a bovine brain calmodulin-binding protein P75 (Sarkar *et al.*, 1984) and its rat homologue P150 (Bregman *et al.*, 1989). MAP2 displays a higher affinity for RIIα (Vallee, 1990) whereas P75 and P150 co-purify with RIIβ and appear to be associated with membranes (Sarkar *et al.*, 1984; Bregman *et al.*, 1989). Other proteins in rat sperm flagellum (Horowitz *et al.*, 1988)

and in bovine brain (Lohmann *et al.*, 1984, 1988) also interact with RII with high affinity.

Recently, the binding domain for MAP2 and P75 has been localized within the first 50 amino acids of RIIβ (Luo *et al.*, 1990) and the requirement of regulatory subunit dimerization for interaction with other proteins has been established (Scott *et al.*, 1990). Conversely, a 31 amino acid N-terminal site in MAP 2A, 2B and 2C (Obar *et al.*, 1990; Rubino *et al.*, 1990), a 15 amino acid C-terminal site in P150 (Bregman *et al.*, 1989) and a 26 amino acid C-terminal segment in P75 (Bregman *et al.*, 1991) were shown to determine the binding of these proteins to RII.

The exact manner in which cAMP-PKII is attached to membranes is not clear. None of the RII amino acid sequences contain transmembrane or membrane anchor sequences (Jahnsen *et al.*, 1986; Scott *et al.*, 1987). Furthermore, the enzyme can be solubilized with Triton X-100 and exhibits properties identical to those of the cytoplasmic enzyme (Corbin *et al.*, 1977; Rubin *et al.*, 1979; Lohmann *et al.*, 1980; Bregman *et al.*, 1989). Both findings have suggested that association of RII with membranes might be mediated by high affinity binding to an integral membrane protein (anchor protein) (Rubin *et al.*, 1979; Bregman *et al.*, 1989).

In this paper we report the identification of a 85 kDa Golgi membrane protein that specifically interacts with RIIβ, the only isoform present in a Golgi-rich fraction prepared from human lymphoblasts (KE37 cell line). We propose that this protein, which exhibits properties of an integral membrane protein, is the anchor which links cAMP-PKII to the Golgi apparatus of human lymphoblasts.

Results

RIIβ but not RIIα is associated with isolated Golgi vesicles in human lymphoblasts

The subcellular localization of RIIα and RIIβ in human lymphoblasts (KE37 cell line) was examined by immunofluorescence using specific polyclonal antibodies against each purified RII isoform. In KE37 cells, the Golgi apparatus is seen as a ring-like structure around the centrosome; both RII isoforms appeared to be concentrated in this Golgi-centrosomal area (shown elsewhere; Keryer,G., Rios,R.M., Lohmann,S.M. and Bornens,M., submitted). Co-localization of both anti-RIIβ antibody and CTR314 monoclonal antibody which specifically recognizes the Golgi apparatus (Bailly *et al.*, 1988) is shown in Figure 1. In order to ascertain which of the RII isoforms is associated with the Golgi apparatus and to identify the RII-binding protein(s) involved in the association between RII and Golgi membranes, we prepared a fraction enriched in Golgi vesicles by flotation on a density sucrose gradient according to Balch *et al.* (1984). To test the enrichment of different fractions in Golgi membranes, proteins from total homogenate (fraction H), nuclear pellet (fraction N), postnuclear supernatant (fraction PNS) and those fractions resulting of the discontinuous sucrose gradient were analysed by SDS−PAGE and transferred to nitrocellulose filters. Filters were then processed by immunolabelling with an affinity purified polyclonal antibody against galactosyl transferase (GT), a well characterized *trans*-Golgi marker (Berger *et al.*, 1981). In agreement with Balch *et al.* (1984), almost all the galactosyl transferase protein was detected in the 35 to 27%

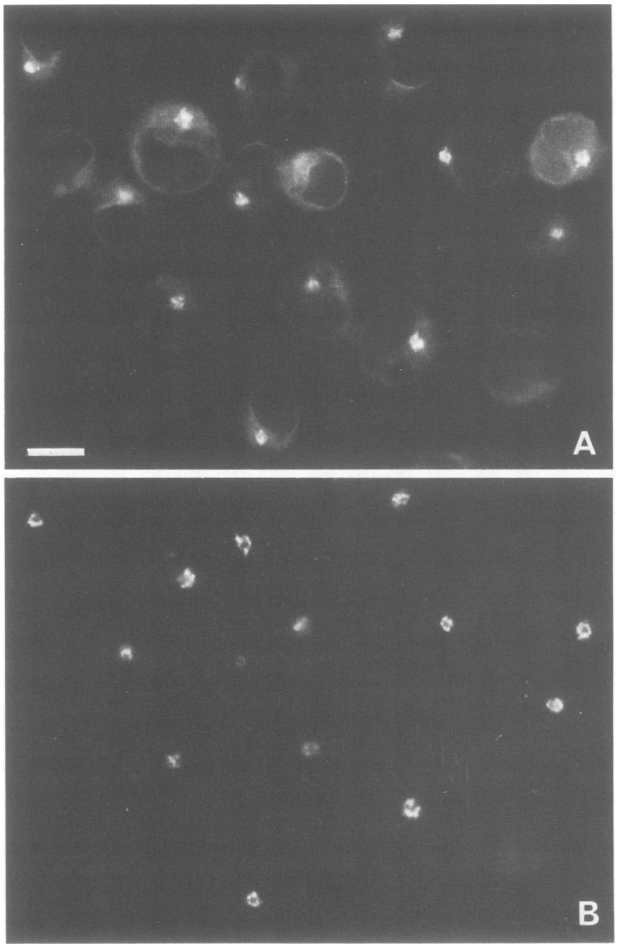

Fig. 1. Indirect immunofluorescence microscopy of KE37 cells stained with anti-RIIβ antibody (**A**) and the monoclonal CTR314 (**B**) that specifically recognizes the Golgi apparatus. Bar = 10 μm.

sucrose gradient interface (not shown). The Golgi-rich fractions were centrifuged as described in Materials and methods and referred to as fraction G in all subsequent experiments. Figure 2A shows the Coomassie stained protein profiles of different subcellular fractions. The protein patterns of H, N and PNS fractions were similar. In contrast, the Golgi-rich fraction showed a protein profile clearly different from the others. In Figure 2B, enrichment of GT in the Golgi fraction as compared with the other subcellular fractions is shown.

The presence of R and C subunits of cAMP-PKII in Golgi vesicles was investigated by immunoblotting using specific polyclonal antibodies against rat heart RIIα, rat brain RIIβ and bovine heart C purified subunits. Both RIIβ and C subunits were highly enriched in the Golgi fraction (Figure 2C). No signal, however, was detected when blots of Golgi vesicles were incubated with an anti-rat heart RIIα antibody (Figure 3). As positive controls, purified bovine heart RIIα and a preparation of bovine brain MAPs that contained RIIα (Theurkauf and Vallee, 1982) were treated in parallel with the same anti-RIIα antibody (Figure 3). A similar result was obtained using an anti-bovine heart RIIα antibody (Pariset *et al.*, 1989; not shown). Therefore, our results indicate that only RIIβ is present in isolated Golgi vesicles of human lymphoblasts.

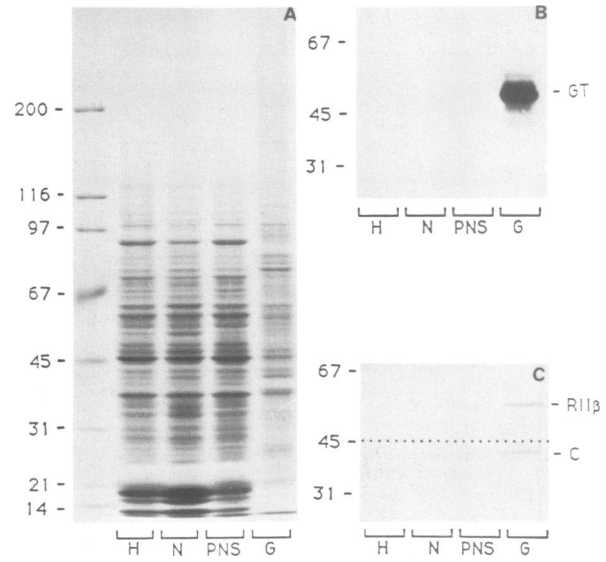

Fig. 2. Co-purification of RIIβ and C subunits of cAMP-PKII with the Golgi marker galactosyl transferase (GT) following fractionation of crude homogenates of KE37 cells on sucrose gradients. (**A**) Coomassie blue stained SDS–PAGE (6 to 15% resolving gel) of different KE37 subcellular fractions. Samples are: lane H, total homogenate; lane N, nuclear pellet fraction; lane PNS, postnuclear supernatant; lane G, Golgi-rich fraction. Lanes H, N and PNS contain 75 μg of protein each; lane G contains 50 μg of Golgi fraction protein. Gels with identical samples to those in (A) were electrophoretically transferred to nitrocellulose filters and the presence of either GT (**B**) or RIIβ and C subunits (**C**) in each fraction was determined by immunoblotting with specific antibodies. Positions of GT, RIIβ and C are indicated on the right. On the left of each figure, the molecular weights of marker proteins given in kDa are indicated: myosin (200), β-galactosidase (116), phosphorylase b (97), bovine serum albumin (67), ovalbumin (44), carbonic anhydrate (31), soybean trypsin inhibitor (21) and lysozyme (14).

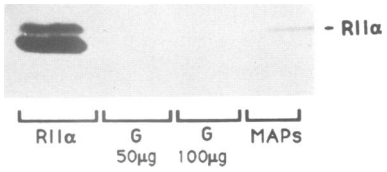

Fig. 3. Purified bovine heart RIIα (0.5 μg), Golgi-rich fraction (50 and 100 μg) and a crude preparation of bovine brain MAPs were subjected to SDS–PAGE, transferred to nitrocellulose and assayed with an anti-rat heart RIIα antibody.

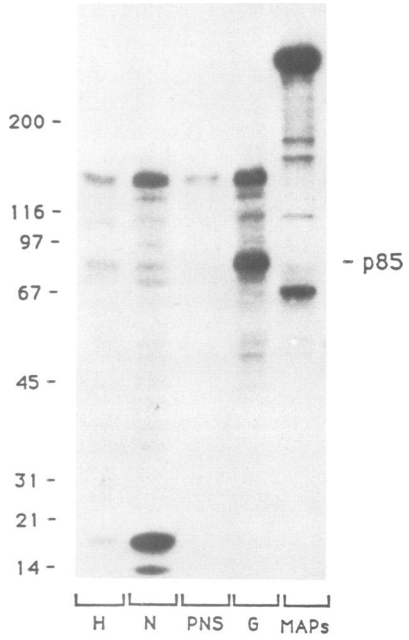

Fig. 4. Autoradiogram showing RII-binding proteins present in different subcellular fractions of KE37 cells as detected by a [^{32}P]RII overlay technique. Proteins of total homogenate (lane H), nuclear pellet (lane N), postnuclear supernatant (lane PNS), Golgi-rich fraction (lane G) and a bovine brain MAPs preparation (as a positive control) were resolved by SDS–PAGE, transferred to nitrocellulose filters and overlaid with ^{32}P-labelled RII. RII was purified and labelled with [γ-^{32}P]ATP as described under Materials and methods. The position of p85 is indicated at right.

Identification of RII-binding proteins in the Golgi-rich fraction

Potential RII-binding proteins in the different subcellular fractions were detected using an RII overlay procedure. This procedure involves labelling of Western blots with *in vitro* phosphorylated RII subunit. Filters containing proteins from total homogenate, nuclear pellet, postnuclear supernatant and Golgi fraction were incubated with [^{32}P]RII from bovine heart, washed and autoradiographed (Figure 4). A similar result was obtained with RII from bovine or rat brain (not shown). A crude preparation of bovine brain MAPs was included as a positive control. In the Golgi fraction, two major bands of M_r 160 000 and 85 000 (designated p160 and p85) showed the ability to bind RII. The p160 protein was also detected in other subcellular fractions, mainly in the nuclear pellet fraction; in contrast, p85 could only be detected in the Golgi fraction. When all the individual fractions from a sucrose gradient were analysed by [^{32}P]RII overlay, p85 was exclusively detected in the galactosyl transferase-rich fraction (not shown). RIIβ present in the Golgi fraction was not revealed by the overlay technique.

In vitro phosphorylation of Golgi vesicles

Most RII-binding proteins have been shown to serve as *in vitro* substrates of the C subunit. We therefore tested for the *in vitro* phosphorylation of p85 by C subunit of cAMP-PKII. Golgi vesicles were incubated for 1 min in the presence of [γ-^{32}P]ATP and different additions to the phosphorylation medium. Autophosphorylation of Golgi vesicles is shown in Figure 5A (lane 1). Addition of 10 μM cAMP to the incubation mixture stimulated the phosphorylation of several high and low molecular weight proteins including an 85 kDa polypeptide (Figure 5A, lane 2). The latter was strongly labelled when the phosphorylation assay contained purified bovine heart C subunit (Figure 5A, lane 3). When Golgi vesicles were incubated with C subunit and PKI, a specific inhibitor of C subunit (Cheng *et al.*, 1986), almost no phosphorylation was observed (Figure 5A, lane 4). It must be noted that the phosphorylation patterns presented in Figure 5A, lanes 1 and 2, correspond to gels autoradiographed for 2 h, while gels were exposed for 5 min to obtain the phosphorylation pattern are shown in lanes 3 and 4. In parallel experiments phosphorylation was maintained for 15 min. In the presence of cAMP or cAMP with purified C subunit, many other proteins became phosphorylated and labelling of p85 comparatively decreased

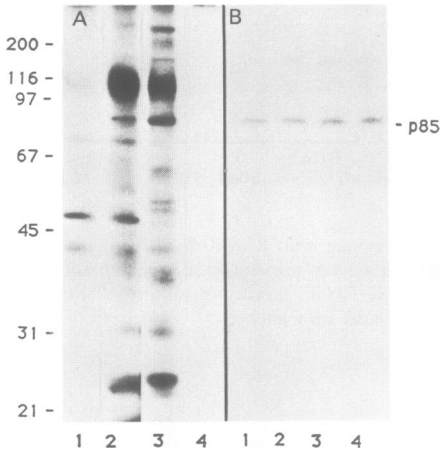

Fig. 5. Phosphorylation of isolated Golgi vesicles. (**A**) Golgi vesicles (50 μg protein) were incubated in the presence of [γ-^{32}P]ATP for 1 min at 25°C and different additions were made to the phosphorylation assay as follows: lane 1, no addition; lane 2, 10 μM cAMP, lane 3, 1 μg of purified bovine heart C subunit; lane 4, 1 μg of C subunit and 0.1 μM PKI. The reaction was stopped by adding SDS sample buffer and the samples were processed for autoradiography. Lanes 1–2, 2 h exposure; lanes 3–4, 5 min exposure. (**B**) Golgi vesicles were phosphorylated as described in (A) but in the presence of unlabelled ATP. Samples were resolved by SDS–PAGE, transferred to nitrocellulose and assayed by [^{32}P]RII overlay. An autoradiogram is shown. Lanes designations are as in (A). The position of p85 is indicated on the right.

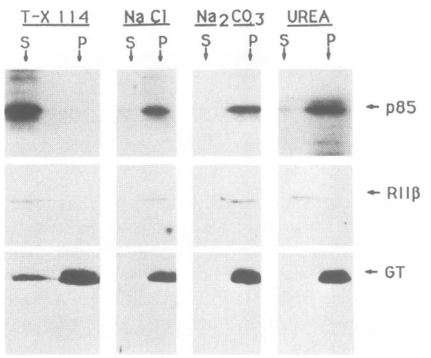

Fig. 6. Different extractions for investigating the topology of p85 in Golgi vesicles. Golgi vesicles (50–75 μg protein) were treated with 2% Triton X-114 (T-X114) as described by Bordier (1981) and both the aqueous (S) and detergent (P) phases were separated by SDS–PAGE and transferred to nitrocellulose filters. Other Golgi vesicles were treated with 1 M NaCl, 200 mM sodium carbonate or 6 M urea and after centrifugation, the resulting supernatants (S) and pellets (P) were processed as described above. Filters were then analysed by [^{32}P]RII overlay to detect p85 (top part) or by immunolabelling with an anti-RIIβ antibody (middle part). Galactosyl transferase, a positive control for a Golgi integral membrane protein, was immunolabelled on duplicate filters (bottom part).

(not shown). To demonstrate that the RII-binding protein p85 and the 85 kDa polypeptide phosphorylated by the C subunit were indeed the same protein, immunoprecipitation experiments were performed (see below).

We further attempted also to determine whether C-dependent phosphorylation of Golgi vesicles could affect the interaction between RII and RII-binding proteins. Golgi vesicles were phosphorylated under the same conditions as described above except that non-labelled ATP was used.

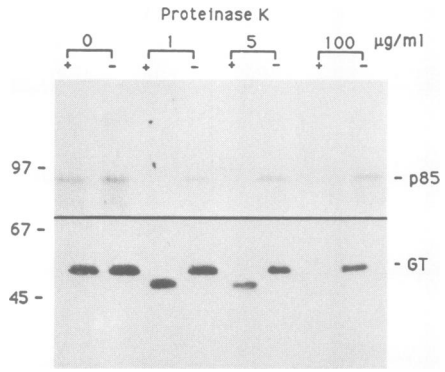

Fig. 7. Effects of increasing concentrations of proteinase K on p85 in isolated Golgi vesicles. Golgi vesicles (50 μg protein) were treated with 0, 1, 5 or 100 μg/ml proteinase K for 30 min at 37°C in the presence (+) or in the absence (−) of 0.4% Triton X-100. After digestion, the samples were resolved by SDS–PAGE, electroblotted on nitrocellulose filters and processed by [^{32}P]RII overlay to identify p85 (top) or by immunolabelling using anti-galactosyl transferase antibody (bottom) as a control.

After phosphorylation, Golgi vesicles were separated by SDS–PAGE, transferred to nitrocellulose and analysed by [^{32}P]RII overlay. As seen in Figure 5B, lanes 1–4, no change in the RII-binding capacity of p85 was observed. Since the RII-binding protein p85 and the 85 kDa polypeptide phosphorylated by C subunit are indeed the same protein (see below), results obtained in this experiment indicate that phosphorylation of p85 by either endogenous or exogenous C subunit did not specifically affect its RII-binding capacity.

Is p85 an integral membrane protein?

We have also analysed the topology of p85 in Golgi vesicles using different extraction procedures and protease protection experiments. First, Golgi vesicles were extracted with Triton X-114, the most commonly employed method for separating hydrophilic and hydrophobic proteins (Bordier, 1981). Samples of the detergent and TCA-precipitated aqueous phases were analysed by [^{32}P]RII overlay (Figure 6). The p85 protein partitioned entirely in the aqueous phase (S). Golgi vesicles were also treated with either (i) 1 M NaCl to remove peripheral membrane proteins; (ii) 0.2 M sodium carbonate pH 11.0 that converts closed vesicles into open sheets releasing vesicles content and peripheral membrane proteins in a soluble form or (iii) 6 M urea in order to dissociate protein complexes. After each incubation, Golgi vesicles were centrifuged and both supernatants (S) and pellets (P) were analysed by [^{32}P]RII overlay (Figure 6, top). In those three conditions tested the protein p85 was found in the pellets.

The nature of the RIIβ–membrane interaction was also examined (Figure 6, middle). RIIβ behaved as p85 after Triton X-114 extraction, 1 M NaCl or 0.2 M sodium carbonate pH 11.0 treatments. However, it was released from membranes by urea treatment. These results indicate that the interaction between RIIβ and the membrane is sensitive to denaturing agents such as urea but resists high salt and high pH treatments.

To control the different extraction procedures, duplicate samples from each treatment were processed for immunolabelling of Western blots with anti-galactosyl transferase antibody (Figure 6, bottom). Galactosyl transferase has been

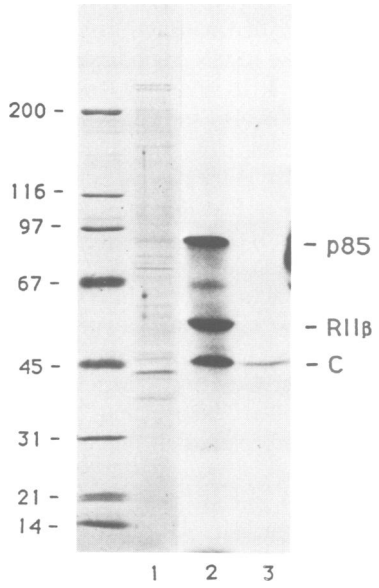

Fig. 8. Immunoprecipitation of the RIIβ−p85 complex. Golgi vesicles were extracted with Triton X-114 and the aqueous phase was subjected to immunoprecipitation with anti-RIIβ antibody or preimmune serum under non-denaturing conditions (see Materials and methods). Both immunoprecipitates were subsequently phosphorylated with purified C subunit in the presence of [γ-^{32}P]ATP as described in Figure 5A, lane 3. After boiling in SDS sample buffer, Sepharose beads were pelleted and the supernatant analysed by SDS−PAGE. lane 1, coomassie blue stained gel of initial Triton X-114 aqueous phase; lane 2, autoradiogram showing the immunoprecipitate phosphorylated by C subunit; lane 3, C subunit phosphorylation of the immunoprecipitate obtained using preimmune serum. Positions of p85, RIIβ and added C are indicated on the right.

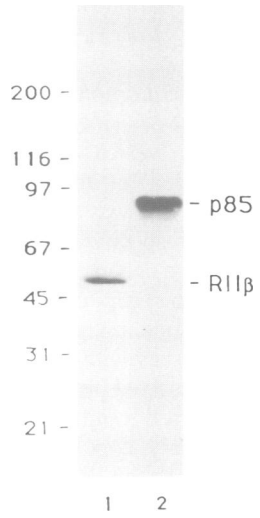

Fig. 9. Purification of RIIβ−p85 complexes by affinity chromatography. A Triton X-114 aqueous phase was prepared from isolated Golgi vesicles and loaded on an agarose−AHA−cAMP affinity column. The column was washed with 2 M NaCl, 10 mM 5′-AMP and 10 mM cGMP as described under Materials and methods. RII−RII-binding protein complexes were eluted by successive washes with 10 mM cAMP. The eluates were collected, concentrated, analysed by SDS−PAGE and transferred to nitrocellulose. Presence of RIIβ in the eluates was confirmed by immunolabelling (lane 1). RII-binding proteins were visualized by [^{32}P]RII overlay (lane 2).

described as a membrane-bound protein with most of its catalytic sites in the lumen of Golgi cisternae (Fleischer, 1981; Strous *et al.*, 1983). It partitioned in the detergent phase when Golgi vesicles were extracted with Triton X-114 and remained associated to membranes after all the other treatments.

Golgi vesicles were also exposed to proteinase K digestion (0, 1, 5 or 100 μg/ml) in the presence or absence of 0.4% Triton X-100 (Figure 7). To monitor the integrity of the Golgi vesicles and the effect of proteinase K treatment, galactosyl transferase digestion was assayed in parallel experiments. As can be seen in Figure 7 (bottom), galactosyl transferase was resistant to proteinase K digestion in the absence of detergent indicating that the membranes formed closed vesicles of the same topographical orientation as *in vivo*. p85 (measured by [^{32}P]RII overlay) was similarly inaccessible to proteinase K in the absence of Triton X-100 (Figure 7, top). However, p85 could not be detected any longer after treatment of the Golgi vesicles with proteinase K in the presence of Triton X-100, indicating that at least the RII-binding site of the protein had been degraded (Figure 7, top). RIIβ itself was degraded by proteinase K in both the presence or absence of detergent (not shown) confirming that it is a peripheral external protein.

Collectively the above data indicate that p85 is an integral membrane protein, except for those results obtained with the Triton X-114 phase separation experiment. A possibility to reconcile these conflicting results would be that p85 is extracted from the membrane as a RIIβ$_2$−p85 complex in which the hydrophilic properties of the RIIβ$_2$ moeity dominates over the hydrophobicity of p85. If this is the case, it should be possible to isolate a native complex RIIβ−p85 from the aqueous phase after Triton X-114 treatment of Golgi vesicles. To test this hypothesis, we attempted to immunoprecipitate the RIIβ−p85 putative complex by an anti-RIIβ antibody, or to co-purify the RII−p85 complex on a cAMP-analogue affinity column, from the Triton X-114 aqueous phase.

Immunoprecipitation of RIIβ−p85 complex

Immunoprecipitation was performed using a rat brain anti-RIIβ antibody under conditions that preserve the integrity of protein complexes but eliminate non-specific interactions, i.e. high detergent and salt concentrations. Protein A−Sepharose beads were coupled to anti-RIIβ antibody IgGs in buffer containing 0.5% NP40, 0.5% DOC and 1.25 M NaCl (2D-NaCl buffer). Golgi vesicles were extracted with Triton X-114 and the aqueous phase adjusted to 2D-NaCl buffer composition from a stock solution. Sepharose beads with bound RIIβ−IgGs were added to this solution, incubated overnight, washed and pelleted. Pellets were then phosphorylated in the presence of [γ-^{32}P]ATP and exogenous C subunit, analysed by SDS−PAGE and autoradiographed. Results of this experiment are shown in Figure 8. Anti-RIIβ antibody immunoprecipitated the RIIβ−p85 complex from the Triton X-114 aqueous phase revealing that both proteins are associated in the Golgi membranes (Figure 8, lane 2). This complex resisted detergents and the high salt concentration, indicating a specific interaction of both proteins. Moreover, this experiment demonstrates that p85 is an *in vitro* substrate of C subunit. Note that here, in the absence of many other

substrates, autophosphorylation of C subunit is prominent as mentioned by Taylor *et al.* (1990).

Purification of RIIβ–p85 complexes by affinity chromatography

After extraction of Golgi vesicles with Triton X-114, the aqueous phase was loaded on an AHA–cAMP agarose affinity column. The column was sequentially washed with 2 M NaCl to release non-specifically bound proteins, with 10 mM 5′-AMP to remove non-cyclic AMP-binding proteins and with cGMP to remove any cGMP-dependent protein kinase. Then RII–RII-binding protein complexes were eluted by successive washes with 10 mM cAMP. Eluates were concentrated and resolved by SDS–PAGE. Gels were silver stained or transferred to nitrocellulose filters. Silver stained gels revealed only a polypeptide of M_r 52 000 which was confirmed to be RIIβ by immunoblotting (Figure 9, lane 1). To detect RII-binding proteins, eluates were processed by [^{32}P]RII overlay (a more sensitive method). Presence of p85 in the eluates was demonstrated as shown in Figure 9, lane 2.

Discussion

This paper reports the presence of both RII and C subunits of cAMP-pKII in a Golgi-rich fraction from KE37 lymphoblastic cells. More precisely, we have demonstrated that only RIIβ is associated with isolated Golgi vesicles from KE37 cells. In discontinuous sucrose density gradients RIIβ migrated as galactosyl transferase, a well known marker of the *trans*-Golgi compartment. Both proteins were found concentrated in the 35 to 27% interface of the gradients. An association of RIIβ with other gradient fractions was not observed. However, contamination of the Golgi fractions by lighter vesicles such as endosomes or prelysosomes cannot be ruled out. Griffiths *et al.* (1990) have reported an association of RII with such vesicles of the endocytic pathway in MDCK cells, and we have initiated ultrastructural immunocytochemical studies at electron microscopy to determine if RIIβ is also associated with these vesicles in KE37 cells.

Only RIIβ, and not RIIα, was shown to be associated with isolated Golgi vesicles. However, additional experiments including Western blotting with specific antibodies, have demonstrated that both isoforms are present in centrosomes isolated from KE37 cells (Keryer,G., Rios,R.M., Lohmann,S.M. and Bornens,M., submitted). It has been shown previously that RII isoforms are differentially expressed in tissues or cells and in tissues containing both isoforms, their expression could be independently regulated by differentiation factors or treatment with hormones or cAMP analogues (Lohmann and Walter, 1984; Jahnsen *et al.*, 1985; Schwartz and Rubin, 1985). Individual differences in expression and in localization of the RII isoforms suggest that they may participate in the regulation of different subcellular processes. RIIβ association with the Golgi membranes suggests that it may mediate cAMP effects on membrane traffic, secretion or subcellular distribution of the Golgi apparatus.

Using different methods, we have identified a novel RII-binding protein, p85, that links RIIβ to the Golgi apparatus. This protein was detected exclusively in a Golgi-rich fraction on Western blots processed by RII overlay, co-immunoprecipitated in a complex with RIIβ from Triton X-114 aqueous extracts of Golgi vesicles treated with an anti-RIIβ antibody, and co-purified with RIIβ on a cAMP analogue affinity column. These results are indicative of a high affinity interaction between RIIβ and p85. In addition to p85, a protein of M_r 160 000 also showed RII binding properties in overlay experiments. p160 was present not only in the Golgi fraction, but also in the nuclear pellet. We have shown recently (Keryer,G., Rios,R.M., Lohmann,S.M. and Bornens,M., submitted) that p160 is present in KE37 centrosomes. Since centrosomes remain associated to the nucleus during subcellular fractionation (Maro and Bornens, 1980) the presence of p160 in the nuclear pellets should not be surprising. p160 also co-sediments with taxol-stabilized microtubules (Keryer,G., Rios,R.M., Lohmann,S.M. and Bornens,M., submitted), suggesting that it could be a microtubule-binding protein that might be able to interact with both membranes and centrosomes.

The physiological role of p85, like for the other RII-binding proteins is unclear. RII-binding proteins are good substrates of the cAMP-dependent protein kinase and may function to maintain a high concentration of the kinase at particular subcellular places near themselves and other important substrates. They may also locate the kinase to microenvironments at which cAMP levels are well regulated by the presence nearby of adenylate cyclase and/or phosphodiesterases. After a 1 min incubation of Golgi vesicles with cAMP and ATP, p85 and about three other polypeptides appeared phosphorylated (see Figure 5A, lane 2). After 15 min phosphorylation many other proteins became phosphorylated but p85 labelling appeared far less prominent. These results strongly suggest, but do not prove, that p85 phosphorylation may be one of the early events following kinase stimulation by cAMP in the Golgi area. Phosphorylation by endogenous C or hyperphosphorylation by exogenous C did not affect the RII-binding capacity of p85. Cyclic AMP-dependent phosphorylation of integral Golgi membrane proteins has been described (Yuan *et al.*, 1987), further indicating a role of cAMP-PK in Golgi activities.

Analysis of p85 topology in Golgi vesicles with standard criteria provided results compatible with p85 being an integral membrane protein although these criteria cannot absolutely demonstrate it. However, partition of p85 into Triton X-114 aqueous phase was anomalous for an integral membrane protein. Other evidence, such as those derived from immunoprecipitation or affinity purification experiments, indicate that p85 exists as a complex with RIIβ and probably C subunits, and therefore we suggest that the hydrophilic properties of this complex may mask p85 hydrophobic nature. The protection of the RII-binding site of p85 from proteolytic action in the absence of detergent suggests that it is not projecting much from the membrane but is rather embedded in lipids. The recent computer aided analysis of secondary structure of four RII-anchoring sequences does not contradict this view (Carr *et al.*, 1991).

It is interesting to consider the possible relationship of p85 with other RII-binding proteins. RIIβ is associated with a 75 kDa bovine brain calmodulin-binding protein (Sarkar *et al.*, 1984) and a 150 kDa related protein in rat brain (Bregman *et al.*, 1989). Both proteins have been shown to be at least partially associated with membranes. In fact, it has been suggested that P150 or P75–RII complexes could be entirely associated to membranes *in situ* (Bregman *et al.*,

1991). The Golgi apparatus was proposed as a potential targeting site for cAMP-PKII type β via P150 in neurons. However, no transmembrane or membrane anchor sequences have been found so far in the partially sequenced P150. Moreover, a polypeptide synthetized from a partial clone of P75 behaves like a soluble protein (Bregman *et al.*, 1989, 1991). Unlike P75, p85 does not bind calmodulin although it has Ca^{2+} binding activity (Rios,R.M., Keryer,G. and Bornens,M., in preparation). Specific antibodies against p85 will be helpful in order to compare p85 with these other RII-binding proteins, including an 80 kDa bovine brain protein described in both soluble and membrane fractions (Lohmann *et al.*, 1984).

Identification and subcellular localization of RII-binding proteins can help to understand cAMP-mediated regulation of cell functions. The diversity of specific kinase effects and the multiple physiological responses to cAMP may in part be generated by the existence of high affinity interactions between RII isoforms and other proteins. So far RII isoforms have been found associated with structures involved in the regulation of cell organization, such as microtubules, centrosomes and the Golgi apparatus. In interphase cells, Golgi apparatus-associated RII appears to be an important anchor for the C subunit which can dissociate under cAMP stimulation and enter the nucleus, while RII remains stationary (Nigg and Hilz, 1985). C subunit phosphorylation of nuclear transcription factors regulates gene expression (Mellon *et al.*, 1989; Büchler *et al.*, 1990; Struthers *et al.*, 1991). Recently, inhibition of C by injection of the protein kinase inhibitor, PKI and co-injection of the cell cycle $p34^{cdc2}$ kinase resulted in nuclear envelope breakdown, an event which marks entry into mitosis (Lamb *et al.*, 1991). In addition, the cAMP-dependent protein kinase has been shown to exist in a complex with the $p34^{cdc2}$ kinase in fibroblasts (Tournier *et al.*, 1991). Both data suggest that the two kinases may be localized near the same substrates, although the timing of their phosphorylation activity may be apposed to one another. Therefore, RII-binding proteins may be of potential significance not only for the regulation of Golgi functions *per se*, but also for the regulation of nuclear and mitotic events. Further investigations concerning how p85 and other RII binding proteins affect kinase anchoring and mobility and their coordination with cellular processes is required.

Materials and methods

Cells, antibodies and purified proteins

The KE37 cell line of T lymphoblastic origin was grown in RPMI 1640 containing 7% fetal calf serum.

The C subunit of cAMP-PK was purified from bovine heart as described by Lohmann *et al.* (1980) and an antibody was produced against it. RII was purified from bovine heart by the method of Corbin *et al.* (1978). Antisera raised against rat heart RIIα and rat brain RIIβ were obtained as described by Lohmann *et al.* (1980), Jahnsen *et al.* (1985, 1986) and Meinecke *et al.* (1990). Each antiserum cross-reacts specifically only with the α or β isoform of human RII (Keryer,G., Rios,R.M., Lohmann,S.M. and Bornens,M., submitted). For immunoblotting experiments, the antibody dilutions routinely used were anti-C 1:400, anti-RIIα 1:200 and anti-RIIβ 1:100. The preimmune serum against RIIβ gave no signal in immunoblot or immunoprecipitation experiments of Golgi proteins. The polyclonal antibody anti-galactosyl transferase (GT) was a kind gift of Dr Berger (Berger *et al.*, 1981) and was used at 1:200 dilution for immunolabelling experiments. The monoclonal antibody CTR314, previously characterized as specific for the Golgi apparatus (Bailly *et al.*, 1988), was used as purified immunoglobulins diluted at 1:1000.

Bovine brain microtubule associated proteins (MAPs) were purified according to Williams and Lee (1982).

Immunofluorescence microscopy

The processing of KE37 cells for immunofluorescence microscopy has been described elsewhere (Bornens *et al.*, 1989; Keryer,G., Rios,R.M., Lohmann,S.M. and Bornens,M., submitted).

Subcellular fractionation

Fractions enriched in Golgi vesicles were isolated by flotation in a sucrose gradient according to Balch *et al.* (1984) with some modifications. Cells were pelleted (300 *g* for 6 min at 20°C) and rinsed in ice-cold phosphate-buffered saline (PBS) 10 mM phosphate buffer, 150 mM NaCl pH 7.2) and then in homogenate buffer (HB) 0.25 M sucrose, 10 mM Tris−HCl pH 7.4). Cell pellets were resuspended in HB containing a mixture of protease inhibitors to achieve a final volume equal to five times the volume of the pellet. The cells were homogenized with 30 pulses in a 15 ml Dounce homogenizer (25−76 μm clearance) and the crude homogenate (fraction H) was centrifuged (9000 *g* for 10 min at 4°C) to produce a nuclear pellet (fraction N) and a postnuclear supernatant (fraction PNS). 6 ml PNS were adjusted to 37% sucrose by addition of a 62% sucrose (w/w) solution containing 10 mM Tris−HCl pH 7.4 and 2 mM Na_2EDTA, loaded into a SW27 tube and overlaid with 15 ml of 35% sucrose (w/w) in 10 mM Tris−HCl pH 7.4 and 10 ml of 27% sucrose (w/w) in the same buffer. Gradients were centrifuged for 2.5 h at 25 000 r.p.m. (85 000 *g*) in a Beckman L855 ultracentrifuge and bands at the 35 to 27% interface were collected in a minimum volume and kept at −80°C. Prior to further processing, Golgi fractions were diluted by addition of several volumes of sucrose-free HB buffer and protease inhibitors were added. The suspensions were centrifuged at 200 000 *g* for 2 h at 4°C and pellets (fraction G) were used for subsequent experiments. Protein concentrations of all samples were determined by Bradford's method (1976) using bovine serum albumin (BSA) as standard.

Electrophoresis and immunoblot analyses

Proteins were separated by SDS−PAGE on 6 to 15% linear acrylamide gradients or 10% acrylamide gels (Laemmli, 1970). Gels were stained with Coomassie blue or electrophoretically transferred to nitrocellulose filters according to Towbin *et al.* (1979). Nitrocellulose filters were blocked for 1 h at 37°C in TBST (10 mM Tris−HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat dry milk. Then filters were incubated overnight at 4°C in the primary antibody diluted in TBST, washed in the same buffer and incubated for 45 min at 37°C with secondary anti-rabbit IgG antibody conjugated to either alkaline phosphatase (Promega, Biotech, Madison, WI) or peroxidase (Catalg, San Francisco, CA). After three washes, alkaline phosphatase activity was revealed by colour development with 0.1 M Tris−HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ containing both nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Peroxidase activity was revealed using the E.C.L. system (Amersham).

Alternatively, nitrocellulose filters were processed for [^{32}P]RII overlay.

[^{32}P]RII overlay technique for detection of RII-binding proteins

[^{32}P]RII overlay was performed as described by Lohmann *et al.* (1988). Nitrocellulose sheets were incubated overnight at 4°C in PBS containing 5% BSA, 5% hemoglobin and 0.25% gelatin (blocking buffer). Purified bovine heart RII (10 μg) was phosphorylated for 1 h in ice in the presence of 50 mM Tris−HCl pH 7.4, 10 mM $MgCl_2$, 20 μM cAMP, 10 mM β-mercaptoethanol, 2.5 μg of purified bovine heart C subunit, 50 μCi of [γ-^{32}P]ATP and 2.5 μM of unlabelled ATP in a final volume of 100 μl. The reaction was terminated by adding 1 mM ATP, 30 mM EDTA and 20 μM cAMP. After saturation, filters were incubated in 15 ml of blocking buffer containing 100 μl of [^{32}P]RII, 0.1% Tween 20 and 0.3% Triton X-100 for 4 h at room temperature. Filters were washed with eight to 10 changes of PBS containing 0.3% Tween 20 and 0.3% Triton X-100 and exposed to Kodak X-OMAT films at −70°C using intensifying screens.

Phosphorylation of Golgi vesicles

Phosphorylation of isolated Golgi vesicles was carried out *in vitro* according to Capasso *et al.* (1985). Golgi vesicles (50 μg protein) were resuspended in 40 μl of STM buffer (0.25 M sucrose, 10 mM Tris−HCl pH 7.4, 1 mM $MgCl_2$). [γ-^{32}P]ATP (5−10 μCi) and non-radioactive ATP were added to give a final concentration of 5 μM ATP in a total volume of 50 μl. Incubation in the phosphorylation medium was for 1 min at 25°C and the reaction was stopped by adding SDS sample buffer. The following additions were made to phosphorylation assays: 10 μM cAMP, 1 μg of purified bovine heart C subunit of cAMP-PK and 0.1 μM of the PKI peptide, an inhibitor of the C subunit (Cheng *et al.*, 1986). Alternatively, Golgi membranes were

phosphorylated under the same conditions with unlabelled ATP, resolved by SDS–PAGE, transferred to nitrocellulose and analysed by [^{32}P]RII overlay.

Membrane extraction and washing procedures

Golgi vesicles (50 μg protein) were extracted with Triton X-114 as described by Bordier (1981). The detergent phase was taken up directly in SDS sample buffer; the aqueous phase was first concentrated by precipitation with 10% (w/v) TCA. Golgi vesicles were also washed with 1 M NaCl, 0.2 M sodium carbonate pH 11.0 (Fujiki *et al.*, 1982) or 6 M urea. After incubation on ice for 30 min, the samples were centrifuged for 1 h at 200 000 *g*. The supernatants (precipitated with 10% TCA) and pellets were resuspended in the same volume of SDS sample buffer and run on 10% acrylamide gels. All samples were analysed by immunoblotting or [^{32}P]RII overlay.

Protease protection experiments

Golgi vesicles were treated with proteinase K using a modification of the method described by Fleischer (1981). Duplicate samples (50 μl) of Golgi vesicles (1 mg protein/ml in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4) were treated for 30 min at 37°C with 0, 1, 5 or 100 μg/ml proteinase K in the presence or absence of 0.4% (v/v) Triton X-100. After addition of 4 mM PMSF, samples were processed for SDS–PAGE followed by either immunoblotting with anti-galactosyl transferase antibody or [^{32}P]RII overlay.

Immunoprecipitation

Golgi vesicles were extracted with Triton X-114 as described above and the aqueous phase was adjusted to 100 mM Tris–HCl pH 8.3, 2 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate and 1.25 M NaCl (2D-NaCl buffer) from a stock solution. 1 μl of either anti-RIIβ antibody or preimmune serum was incubated for 1 h at 4°C with 50 μl of a 50% suspension of Protein A–Sepharose (Pharmacia) in 2D-NaCl buffer. Beads were washed three times with 2D-NaCl buffer before adding 50 μl of the Triton X-114 aqueous supernatant. The mixture was rotated overnight at 4°C and washed five times with 2D-NaCl buffer. After two final washes with 10 mM Tris–HCl pH 7.4, the immunoprecipitates were phosphorylated with purified bovine heart C subunit under the same conditions described above. Phosphorylation was stopped by adding SDS sample buffer. Beads were boiled and centrifuged, and the supernatants analysed by SDS–PAGE and autoradiography.

Purification of RII–RII-binding protein complexes by affinity chromatography

A Triton X-114 aqueous phase was prepared from Golgi vesicles (200 μg protein) and loaded onto a 2.5 ml AHA–cAMP–agarose affinity column (Sigma) previously equilibrated with PEMT buffer (10 mM phosphate buffer pH 6.8, 1 mM EDTA, 15 mM β-mercaptoethanol and 20 U of Trasylol/ml) containing 0.1 M NaCl. After loading, the column was sequentially washed with 2 M NaCl, 10 mM 5′-AMP and 10 mM cGMP in the same buffer before elution with 10 ml of PEMT buffer containing 10 mM cAMP. After concentration, the eluate was analysed by SDS–PAGE. Gels were electrophoretically transferred to nitrocellulose and the blots processed for either [^{32}P]RII overlay or immunoblotting.

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References

Balch,W.E., Dunphy,W.G., Braell,W.A. and Rothman,J.E. (1984) *Cell*, **39**, 405–416.

Bailly,E., Berger,J., Bordes,N., Celati,C. and Bornens,M. (1988) *Eur. Symp. Struct. Funct. Cytoskel.*, **87**, 143.

Beebe,S.J., Oyen,O., Sandberg,M., Froysa,A., Hansson,V. and Jahnsen,T. (1990) *Mol. Endocrinol.*, **4**, 465–475.

Berger,E.G., Mandel,T. and Schilt,U. (1981) *J. Histochem. Cytochem.*, **29**, 364–370.

Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.

Bornens,M., Paintrand,M. and Celati,C. (1989) *J. Cell Biol.*, **109**, 1071–1083.

Bradford,M. (1976) *Anal. Biochem.*, **72**, 248–254.

Bregman,D.B., Bhattacharyya,N. and Rubin,C.S. (1989) *J. Biol. Chem.*, **264**, 4648–4656.

Bregman,D.B., Hirsch,A.H. and Rubin,C.S. (1991) *J. Biol. Chem.*, **266**, 7207–7213.

Büchler,W., Meinecke,M., Chakraborty,T., Jahnsen,T., Walter,U. and Lohmann,S.M. (1990) *Eur. J. Biochem.*, **188**, 253–259.

Cadd,G. and McKnight,G.S. (1989) *Neuron*, **3**, 71–79.

Capasso,J.M., Abeijon,C. and Hirschberg,C.B. (1985) *J. Biol. Chem.*, **260**, 14879–14884.

Carr,D.W., Stofko-Hahn,R.E., Fraser,I.D.C., Bishop,S.M., Acott,T.S., Brennan,R.G. and Scott,J.D. (1991) *J. Biol. Chem.*, **266**, 14188–14192.

Cheng,H.C., Kemp,B.E., Pearson,R.B., Smith,A.J., Misconi,L., Van Patten,S.M. and Walsh,D.A. (1986) *J. Biol. Chem.*, **261**, 989–992.

Clegg,C.H., Cadd,G.G. and McKnight,G.S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3703–3707.

Corbin,J.D., Sudgen,P.H., Lincoln,T.M. and Keely,S.L. (1977) *J. Biol. Chem.*, **252**, 3854–3861.

Corbin,J.D., Sudgen,P.H., West,L., Flockhart,D.A., Lincoln,T.M. and McCarthy,D. (1978) *J. Biol. Chem.*, **253**, 3997–4003.

De Camilli,P., Moretti,M., Denis Donini,S., Walter,U. and Lohmann,S.M. (1986) *J. Cell Biol.*, **103**, 189–203.

Erlichman,J., Sarkar,D., Fleischer,N. and Rubin,C.S. (1980) *J. Biol. Chem.*, **255**, 8179–8184.

Fleischer,B. (1981) *J. Cell Biol.*, **89**, 246–255.

Fujiki,Y., Hubbard,A.L., Fowler,S. and Lazarow,P.B. (1982) *J. Cell Biol.*, **93**, 97–102.

Griffiths,G., Hollinshead,R., Hennings,B.A. and Nigg,E.A. (1990) *J. Cell Sci.*, **96**, 691–703.

Horowitz,J.A., Wasco,W., Leiser,M. and Orr,G.A. (1988) *J. Biol. Chem.*, **263**, 2098–2104.

Jahnsen,T., Lohmann,S.M., Walter,U., Hedin,L. and Richards,J.S. (1985) *J. Biol. Chem.*, **260**, 15980–15987.

Jahnsen,T. *et al.* (1986) *J. Biol. Chem.*, **261**, 12352–12361.

Lamb,N.J.C., Cavadore,J.C., Labbé,J.C., Maurer,R.A. and Fernandez,A. (1991) *EMBO J.*, **10**, 1523–1533.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lee,D.C., Carmichael,D.F., Krebs,E.G. and McKnight,G.S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3608–3612.

Lohmann,S.M. and Walter,U. (1984) *Adv. Cyclic Nucleotide Res.*, **18**, 63–117.

Lohmann,S.M., Walter,U. and Greengard,P. (1980) *J. Biol. Chem.*, **255**, 9985–9992.

Lohmann,S.M., De Camilli,P., Einig,I. and Walter,U. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6723–6727.

Lohmann,S.M., De Camilli,P. and Walter,U. (1988) *Methods Enzymol.*, **159**, 183–193.

Luo,Z., Shafit-Zagardo,B. and Erlichman,J. (1990) *J. Biol. Chem.*, **265**, 21804–21810.

Maro,B. and Bornens,M. (1980) *Biol. Cell*, **39**, 287–290.

Mellon,P.L., Clegg,C.H., Correll,L.A. and McKnight,G.S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4887–4891.

Meinecke,M., Buchler,W., Fischer,L., Lohmann,S.M. and Walter,U. (1990) In Jeserich,G. *et al.* (eds) *Cellular and Molecular Biology of Myelinisation*. Springer Verlag, Heidelberg, pp. 201–215.

Nigam,S.K. and Blobel,G. (1989) *J. Biol. Chem.*, **264**, 16927–16932.

Nigg,E.A. and Hilz,H. (1985) *EMBO J.*, **4**, 2801–2806.

Nigg,E.A., Schafer,G., Hilz,H. and Eppenberger,H.M. (1985) *Cell*, **41**, 1039–1051.

Obar,R.A., Dingus,J., Bayley,H. and Vallee,R.B. (1990) *Neuron*, **3**, 639–645.

Pariset,C., Feinberg,J., Dacheux,J.L., Oyen,O., Jahnsen,T. and Weinman,S. (1989) *J. Cell Biol.*, **109**, 1195–1205.

Rubin,C.S., Rangel-Adao,R., Sarkar,D., Erlichman,J. and Fleischer,N. (1979) *J. Biol. Chem.*, **254**, 3797–3805.

Rubino,H.M., Dammerman,M., Shafit-Zagardo,B. and Erlichman,J. (1990) *Neuron*, **3**, 631–638.

Sarkar,D., Erlichman,J. and Rubin,C.S. (1984) *J. Biol. Chem.*, **259**, 9840–9846.

Schwartz,D.A. and Rubin,C.S. (1985) *J. Biol. Chem.*, **260**, 6296–6303.

Scott,J.D., Glaccum,M.B., Zoller,M.J., Uhler,M.D., Helfman,D.M., McKnight,G.S. and Krebs,E.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5192–5196.

Scott,J.D., Stofko,R.E., McDonald,J.R., Comer,J.D., Vitalis,E.A. and Mangili,J.A. (1990) *J. Biol. Chem.*, **265**, 21561–21566.
Stein,J.C., Farooq,M., Norton,W.T. and Rubin,C.S. (1987) *J. Biol. Chem.*, **262**, 3002–3006.
Strous,G.J., Van Kerkhof,P., Willemsen,R., Geuze,H.J. and Berger,E.G. (1983) *J. Cell Biol.*, **97**, 723–727.
Struthers,R.C., Vale,W.W., Arias,C., Sawchenko,P.E. and Montminy,M.R. (1991) *Nature*, **350**, 622–624.
Taylor,S.S., Buechler,J.A. and Yonemoto,W. (1990) *Annu. Rev. Biochem.*, **59**, 971–1005.
Theurkauf,W.E. and Vallee,R.B. (1982) *J. Biol. Chem.*, **257**, 3284–3290.
Tournier,S., Raymond,F., Gerbaud,P., Lohmann,S.M., Doree,M. and Evain-Brion,D. (1991) *J. Biol. Chem.*, **266**, 19018–19022.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Uhler,M.D., Chrivia,J.C. and McKnight,G.S. (1986a) *J. Biol. Chem.*, **261**, 15360–15363.
Uhler,M.D., Carmichael,D.F., Lee,D.C., Chrivia,J.C., Krebs,E.G. and McKnight,G.S. (1986b) *Proc. Natl. Acad. Sci. USA*, **83**, 1300–1304.
Vallee,R.B. (1990) *Cell Motil. Cytoskel.*, **15**, 204–209.
Williams,R.C. and Lee,J.C. (1982) *Methods Enzymol.*, **85**, 376–385.
Yuan,L., Barriocanal,J.G., Bonifacino,J.S. and Sandoval,I.V. (1987) *J. Cell Biol.*, **105**, 215–227.

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