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Clathrin β-light chain of rat liver coated vesicles is phosphorylated in vitro and in vivo

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Clathrin β-light chain of rat liver coated vesicles is phosphorylated in vitro in the presence of poly(L-lysine) by an endogenous protein kinase which appears to be similar to casein kinase II. Clathrin β-light chain is also phosphorylated in vivo. After injection of [^{32}P]phosphate into rats and preparation of purified coated vesicles in the presence of phosphatase inhibitors, electrophoretic analysis showed the presence of several labeled polypeptides including clathrin β-light chain. A polypeptide of 50 kDa, which may correspond to the major polypeptide phosphorylated in vitro of coated vesicles, is also labeled in vivo.

Coated vesicle; Poly(L-lysine); Clathrin β-light chain; Casein kinase II; Protein phosphorylation

1. INTRODUCTION

Coated vesicles (CVs) play an important role in intracellular transport either in the internalization of external molecules and their receptors or in protein maturation and secretion processes [1,2]. Each transport cycle between membrane-bound compartments of eukaryotic cells requires coordinated coating and uncoating of the membrane by clathrin and its associated proteins. CVs are cage-like structures consisting of a membrane enclosed by a polyhedral protein lattice composed of clathrin, a 180 kDa polypeptide, associated with 36 and 33 kDa polypeptides: the clathrin light chains. Minor polypeptides of 100 and 50 kDa have also been reported to be associated with the clathrin coat [3–5]. The phosphorylation of CV polypeptides was first reported in vitro in calf brain by Pauloin et al. [6], then confirmed in different tissues including rat liver, showing essentially the same patterns. The major phosphopolypeptide observed has a molecular mass of 50 kDa but several other proteins are also phosphorylated [7,8]. Pauloin and Jolles [9] have shown that in brain CVs, phosphorylation of the 50 kDa polypeptide is strongly stimulated in the presence of clathrin light chains. More recently, it has been shown that the clathrin β-light chain (33 kDa) is phosphorylated in vitro in the presence of polylysine or histones in calf brain CVs [10,11]. The enzyme responsible for this phosphorylation has been identified as casein kinase (CK) II in calf brain [12]. The light chain of clathrin CVs displays a heterogeneous structure in different tissues or species [13]. Here, we have examined whether this chain is phosphorylated in other tissues than brain. We show that the β-light chain of clathrin is phosphorylated in vitro in CVs of rat liver. This phosphorylation seems to be a physiological function of CVs, since it is observed in vivo: a labeled band corresponding to the β-light chain of clathrin is observed after PAGE of liver CVs prepared from rats injected with ^{32}P.

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Abbreviations: Mes, (2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PNPP, *p*-nitrophenyl phosphate, disodium salt; NEM, *N*-ethylmaleimide; PCA, perchloric acid

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2. MATERIALS AND METHODS

2.1. *Materials*

Buffalo rats were obtained from IRSC (Villejuif). ^2H_2O was obtained from CEA (Saclay, France); EGTA, Mes, Tris, NEM, PMSF, casein, heparin, histone, poly(L-lysine) (HBr, M_r 17000), and unlabeled nucleotides were purchased from Sigma. EDTA and MSH were from Merck. [γ-^{32}P]ATP (spec. act. 3000–5000 mCi/mmol) was from Dupont (New England Nuclear). Acrylamide and other reagents for electrophoresis were from Biorad.

2.2. *Purification of CVs*

CVs were prepared from rat liver by a modification of the method of Nandi et al. [14]. All procedures were carried out at 4°C. Livers from rats were excised, washed and homogenized in 2 vols of 100 mM Mes buffer containing 250 mM sucrose, 1 mM EGTA, 1 mM PMSF, 0.5 mM $MgCl_2$ adjusted to pH 6.5 (buffer A). The homogenate was centrifuged at 20000 × *g* for 50 min in a 60 Ti rotor. The supernatant was centrifuged at 100000 × *g* for 60 min. The resulting pellet was homogenized in buffer A, centrifuged for 10 min at 10000 × *g* and the supernatant centrifuged for 60 min at 140000 × *g*. This procedure was repeated once. The final pellet was resuspended in buffer A and the supernatant obtained after the low-speed centrifugation as above was layered over 8% sucrose/^2H_2O solution (with the same buffer salts as buffer A), then centrifuged for 2 h at 80000 × *g* at 20°C in an SW41 rotor. The pelleted fraction was resuspended in buffer A, homogenized and centrifuged for 10 min at 20000 × *g*. The supernatant containing purified CVs was stored at −80°C in aliquots. The purity of CV preparation was monitored by means of their relative content of clathrin (70% of total proteins) after SDS-PAGE and by electron microscopy analysis (fig.1a,b).

2.3. *In vivo labeling of CVs*

3 mCi Na [^{32}P]phosphate (Oris, France) was injected intraperitoneally into 12-week-old Buffalo rats. 2 h later animals were killed by decapitation and livers were excised, washed and homogenized in 2 vols buffer A without $MgCl_2$ and containing phosphatase inhibitors: 10 mM PNPP, 20 mM EDTA, 10 mM Na, K tartrate, 25 mM NEM, 25 mM Na pyrophosphate, 50 mM NaF and 10 mM orthovanadate (buffer B). The homogenate was then processed as described above. The cytosol fraction enriched in CVs was then boiled for 10 min and the supernatant containing the

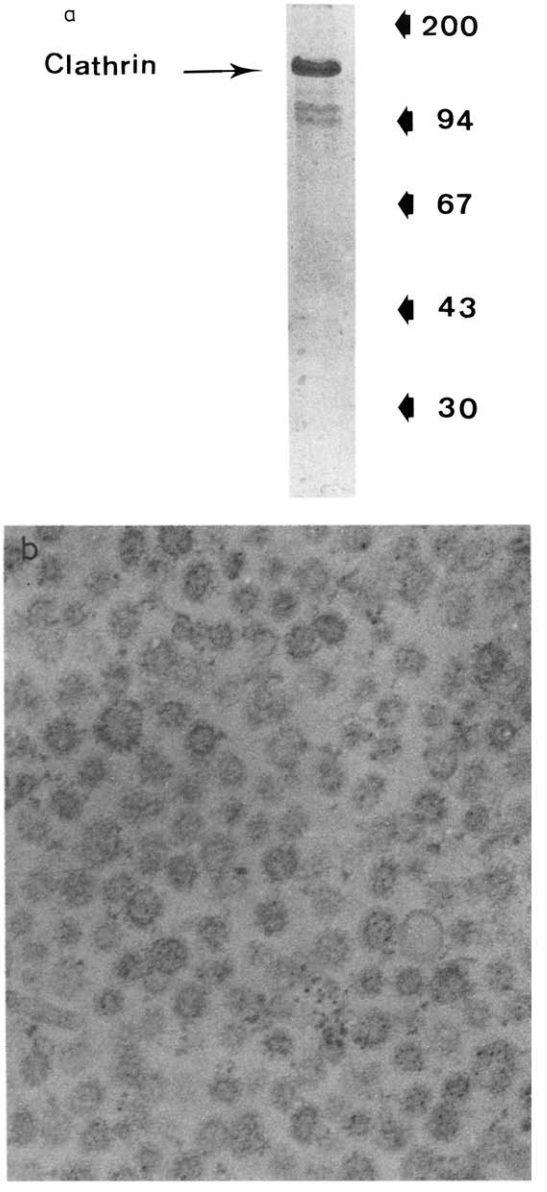

Fig.1. Electrophoretic pattern (a) and electron microscopy (b) of rat liver purified CVs. CVs purified as described in section 2 were analyzed by SDS-PAGE and electron microscopy (by courtesy of Dr Puvion, IRSC, Villejuif).

clathrin β-light chain was submitted to SDS-PAGE as described below.

2.4. *Preparation of casein kinases*

CK II was prepared according to Meggio et al. [15]. CK I was prepared as described by Pierre and Loeb [16].

2.5. *In vitro phosphorylation assay*

The standard phosphorylation assay involved incubation of 20 μg CVs at 33°C with 0.5 μCi of 10 μM [γ-^{32}P]ATP in 50 mM Tris-HCl buffer (pH 7.4), containing 10 mM $MgCl_2$ in a total volume of 35 μl for 3 min. Reactions were stopped by addition of 27 μl Laemmli sample buffer [13] containing 20% Triton X-100. After incubation for 40 min at room temperature, samples were loaded on polyacrylamide slab gels containing 15% acrylamide, 0.1% bisacrylamide, 0.1% SDS according to Beemon and Hunter [17]. Electrophoresis was carried out at 4°C. Gels were stained with Coomassie blue 250, dried and submitted to autoradiography using X-ray film at −70°C. Protein content of samples was determined according to Bradford [18].

3. RESULTS AND DISCUSSION

3.1. *In vitro phosphorylation*

The addition of polylysine (50 μg/ml) to highly purified rat liver CVs together with Mg^{2+} (10 mM) and [γ-^{32}P]ATP promotes the phosphorylation of a 33 kDa polypeptide (fig.2a, lanes A,B). To a lesser extent, the same result is obtained in the presence of histone H1, which contains numerous lysine residues (lane C). We have identified the 33 kDa polypeptide as the clathrin β-light chain by the following criteria: M_r in SDS-PAGE, stability against boiling [11] (fig.2b) and association with the triskelions: when CVs, phosphorylated in the presence of polylysine, are dissociated in 1 M Tris (pH 8.6), the 33 kDa polypeptide is found after centrifugation in the supernatant fraction which contains the triskelions of clathrin; furthermore, after two-dimensional electrophoresis of CVs, no other polypeptide is found in the 33 kDa region (not shown).

A casein kinase activity is present in rat liver CVs, as shown in fig.2. When CVs are incubated in the presence of exogenous casein, significant

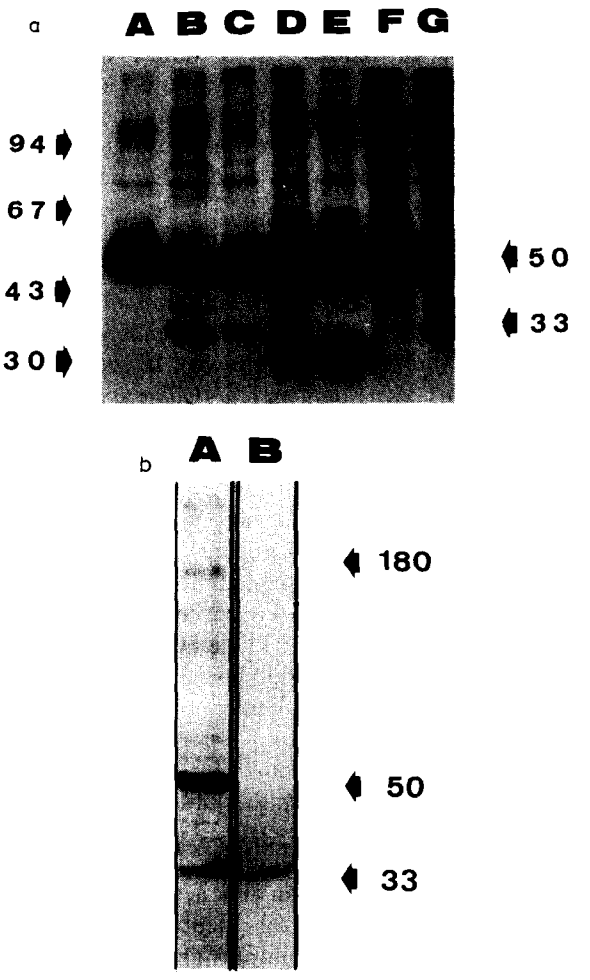

Fig.2. (a) In vitro phosphorylation of 50 and 33 kDa CV polypeptides. 20 μg CVs were incubated as described in section 2 without (A) or with: 50 μg/ml of polylysine (B), 200 μg/ml of histone H1 (C), 1 mg/ml of casein without (D) or with 50 μg/ml of polylysine (E), 0.5 μg CK II without (F) or with 0.5 μg polylysine (G). (b) Thermostability of the 33 kDa ^{32}P-labeled polypeptide. 20 μg CVs were phosphorylated as described in section 2, boiled for 5 min (A) and centrifuged to remove denatured proteins. Electrophoresis and autoradiography of the supernatant is shown in B.

phosphorylation of this protein is observed (lane D). This phosphorylation is increased in the presence of polylysine (lane E), suggesting that this kinase may be the CK II which is stimulated by polycations. The addition of purified CK II to CVs, in the presence of polylysine, provokes strong phosphorylation of the 33 kDa polypeptide

(lane G) which is much stronger than that in the absence of exogenous CK II (lanes B,C). The presence of polylysine is an absolute requirement for this phosphorylation. If no polylysine is added, even in the presence of exogenous CK II, only weak phosphorylation of the 33 kDa polypeptide is detectable (lane F).

Inhibitors of CK II are also potent inhibitors of clathrin β-light chain phosphorylation, as well as cold GTP (5 mM) or heparin (20 μg/ml). Moreover, CK I and cAMP-dependent protein kinase are ineffective (not shown). It appears that as in brain CVs [12], it is CK II-like activity present in CVs which phosphorylates the β-light chain of clathrin. Polylysine acts probably through interaction with the substrates, as suggested for other systems by Hathaway and Traugh [19]. When casein in excess (1 mg/ml) is added to CVs in the presence of polylysine, stimulation of the casein phosphorylation is observed but no phosphorylation of the 33 kDa polypeptide is seen, suggesting that all the polylysine has been complexed with casein (lane E).

3.2. *In vivo phosphorylation*

As with every phosphorylation observed in vitro, phosphorylation of the 33 kDa polypeptide may be artificial. CK II is found in CVs but its presence may be due to an artefact of the preparation or the conditions of incubation (pH 7.5, 10 mM Mg^{2+}), although improbable, may not correspond to the physiological condition of CVs in the cell.

In order to ascertain that this phosphorylation is physiological, Na[^{32}P]phosphate was injected into rats. Liver CVs were purified in the presence of phosphatase inhibitors as described in section 2, and phosphorylation of CV polypeptides was analyzed by PAGE.

Fig.3 shows that among several labeled polypeptides, a 33 kDa polypeptide is clearly visible (lane A). This species is heat stable, since it is present in the supernatant of CVs after boiling (lane B), suggesting strongly that it is the β-light chain of clathrin (see section 3.1). It has been reported that light chains may contain tightly bound nucleotides [20]. Treatment of the gels with hot PCA, which hydrolyses nucleotides, eliminates the possibility that labeling of the 33 kDa polypeptide is associated with nucleotides. The 110 kDa protein,

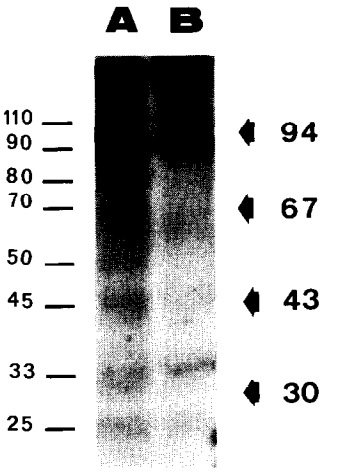

Fig.3. In vivo phosphorylation of rat liver CVs. Liver CVs were purified from ^{32}P-injected rats and CVs were analyzed by SDS-PAGE. After Coomassie blue staining, the gels were further incubated in 5% PCA at 95°C for 15 min in order to hydrolyze nucleic acids and nucleotides which can contaminate CVs.

which is part of a complex also containing the 50 kDa protein that is the major protein phosphorylated in vitro (fig.2), has been shown to be phosphorylated in vivo in CVs of cultured rat neurons [21]. A band corresponding precisely to a 50 kDa protein is clearly visible, but further identification is necessary. A strongly phosphorylated protein of 90 kDa is partially thermostable and remains to be identified as well as the 80, 70, 45 and 25 kDa proteins.

The addition of several protein phosphatase inhibitors to buffer during the preparation of CVs is an essential requirement for obtaining efficient in vivo phosphorylation of CV polypeptides. When in vivo phosphorylation experiments were carried out in the presence of only NaF, the most common phosphatase inhibitor, no reproducible labeling of the polypeptides described above was observed. On the other hand, very rapid dephosphorylation activity takes place in vitro when rat liver CVs are incubated in the presence of liver homogenate or cytosol prepared in the absence of phosphatase inhibitors (fig.4, lanes A–C). Several known phosphatase inhibitors have been assayed. The results show (fig.4, lanes D–F) that the most efficient inhibition of phosphatase activities was obtained by using a complete cocktail of inhibitors as described in section 2.

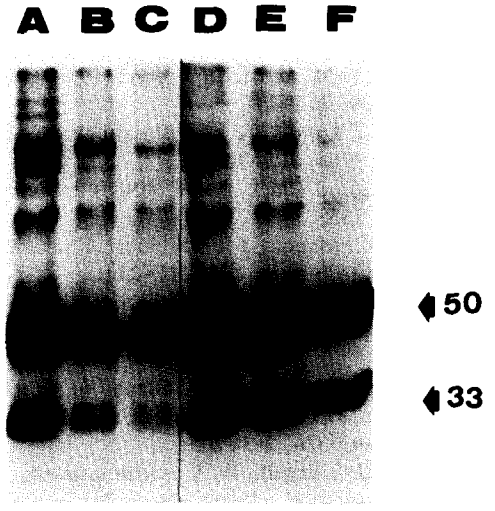

Fig.4. In vitro dephosphorylation of rat liver CVs by cytosol. Rat liver CVs were phosphorylated in vitro as described in section 2. Cold ATP (10^{-3} M) was added after 3 min incubation. This masks, by isotopic dilution of labeled [γ-^{32}P]ATP, subsequent phosphorylation permitting one to follow dephosphorylation. At the same time, cytosol [1 mg/ml of protein in either buffer A (standard CV preparation buffer)] or buffer B [containing phosphatase inhibitors (see section 2)] was added and aliquots were pipetted after 0, 5 and 15 min and submitted to SDS-PAGE analysis. Lanes – (A–C) buffer A: 0, 5, 15 min; (D–F) buffer B: 0, 5, 15 min.

3.3. *Conclusion*

In summary, we have shown for the first time that clathrin β-light chain is phosphorylated in vivo. Our results show that phosphorylation of the observed 33 kDa polypeptide in vitro, in brain CVs [10–12] as well as in rat liver, as demonstrated here, corresponds to a physiological reality. In vitro, β-light chain is phosphorylated by a CK II present in CVs. It is likely to be the same in vivo. Analysis of the sites phosphorylated in vivo and in vitro would be required to demonstrate this fact. In brain, β-light chain is phosphorylated in vitro on serine residues [12]. CK II phosphorylates preferentially acidic amino acid sequences of type SEEE: serine followed by a cluster of acidic amino acids such as glutamic acid [22]. The complete amino acid sequence of the β-light chain of clathrin from brain and lymphocytes has been recently determined [23]. Moreover, the amino acid sequence of exposed region of the β-light chain has been also determined in native triskelions and CVs [24]. From these results, one may suggest that the Ser 188, followed by two glutamic acids, can be the phosphorylation site, since it is located in the exposed region (158–208) of the β-light chain and that this sequence is common to brain and other tissues. The β-light chain from brain seems to be different from light chains from other tissues. Phosphorylation of the β-light chain could correspond to a functional difference between triskelions and β-light chains, respectively. Clathrin heavy chains (180 kDa) are strongly conserved between species whereas a number of variants of clathrin light chains (33 and 36 kDa) have been reported [13]. The fact that in both calf brain and rat liver, the 33 kDa clathrin β-light chain, in spite of its variability, is phosphorylated by the same kinase favors a possible physiological significance of this phosphorylation.

The light chains have been proposed as playing a regulatory role in CV function. They stimulate phosphorylation of the 50 kDa protein [9] and are required for activity of the uncoating ATPase [25]. They have also been conjectured as modulating clathrin interaction with membranes [26,27]. Further investigation will elucidate whether phosphorylation of the β-light chain may modify one or several of these roles.

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