

LABELING OF SPECIFIC PROTEINS IN RAT OVARIAN PLASMA MEMBRANES WITH [γ - 32 P]GTP

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1. Introduction

Guanosine 5'-triphosphate (GTP) has been implicated in a number of biological processes associated with protein synthesis [1], microtubular function [2,3] and in the control exerted by a large number of hormones [4]. Our interest in plasma membrane-associated reactions that specifically utilize GTP has been stimulated by the recognition that this nucleotide is directly involved in the control of adenylate cyclase activity [4] and by the demonstration of specific binding sites for GTP in plasma membranes of hepatocytes and adipocytes [5]. A hormone-sensitive GTPase in turkey erythrocytes [6] and a GTP-binding protein associated with pigeon erythrocyte adenylate cyclase have been described [7].

We undertook a search for GTP-dependent phosphorylation in ovarian membranes since in vitro desensitization of LH-sensitive adenylate cyclase has been postulated to involve phosphorylation reactions [8,9]. This desensitization process in the rat ovary is dependent on low concentrations of GTP even in the presence of ATP [10–12]. Similar findings have been made also in normal rat kidney cells [13]. Here we report evidence that [γ - 32 P]GTP preferentially labels two proteins in rat ovary and parotid membranes [14] that differ structurally from the proteins that are substrates for ADP-ribosylation by cholera toxin [15–17] and which are thought to be involved in the regulation of adenylate cyclase by GTP.

Abbreviations: LH, luteinizing hormone; PAGE, polyacrylamide gel electrophoresis; PMSF, phenyl methyl sulfonyl fluoride; p(NH)ppA, adenosine 5'-(β , γ imido) triphosphate; p(NH)ppG, guanosine 5'-(β , γ imido) triphosphate; SDS, sodium dodecyl sulfate

2. Materials and methods

[γ - 32 P]ATP (>2000 Ci/mmol), [γ - 32 P]GTP (10–30 Ci/mmol) and a mixture of [14 C]methylated protein markers were purchased from the Radiochemical Centre, Amersham. Cholera toxin was obtained from Schwarz/Mann. Nicotinamide adenine dinucleotide (NAD) pyrophosphorylase and hexokinase were from Boehringer. CTP, p(NH)ppA, β -nicotinamide mononucleotide (NMN), *n*-octyl glucoside, PMSF, cholic acid, Triton X-100 and Lubrol PX were obtained from Sigma. *Staphylococcus aureus* V8 protease (36–900) was obtained from Miles Labs. All other reagents were as in [12].

Rat ovarian plasma membranes were prepared as in [18]. Rat parotid membranes were prepared according to [19], and human erythrocyte ghosts according to [20]. Protein determination was according to [21], using bovine serum albumin as standard.

Labelling of membranes with [γ - 32 P]GTP was as follows: The incubation mixture (final vol. 1.5 ml) contained 25 mM Tris-acetate (pH 7.4), 5.0 mM Mg-acetate, 1 mM DTT, 10 μ M PMSF and plasma membranes (100–160 μ g). The reaction was initiated by the addition of [γ - 32 P]GTP (2 – 4×10^7 cpm; 0.5–1.2 μ M). Incubation was at 30°C for 2 min. The reaction was terminated by immersion of test tubes in ice (4°C) and centrifugation 30 min at 100 000 \times g. The resulting pellets were frozen at –20°C. Membrane pellets were prepared for PAGE by resuspension in a solution containing: 0.7% Triton X-100, 0.5 mM Hepes (pH 8.0), 0.25 mM Mg-acetate, 0.125 mM EDTA and 10 μ M PMSF. The solution was allowed to stand on ice for 1 h.

[32 P]NAD was prepared according to [15]. Activation of cholera toxin was according to [22]. Labelling

of ovarian plasma membranes with [32 P]NAD and activated cholera toxin was performed according to [16], and membranes were extracted with either 0.7% Lubrol PX or 0.7% Triton X-100.

Prior to electrophoretic separation protein samples were incubated in 3% SDS and 5% mercaptoethanol for 1 h at 60°C. SDS-PAGE was performed on slabs (1.6 mm \times 13 cm \times 18 cm) according to [23] with 7.5–15% acrylamide gradients. Separation was accomplished at constant voltage (120 V) for 14–16 h. Gels were dried and exposed for 6 days to X-ray film (Agfa Gevaert RP.2) in the presence of intensifying screens (DuPont, Cronex, Quanta II) at -70°C. Where indicated, proteins were stained with Coomassie blue.

3. Results and discussion

Incubation of ovarian plasma membranes with [γ - 32 P]GTP for ≤ 2 min resulted in the labeling of a single protein as demonstrated on SDS-PAGE (fig.1A, lane 1). Designated band 'a', this protein had an app. M_r 33 000 \pm 300 (SEM, $n = 11$). Incorporation of label into band a was abolished upon addition of 1 mM unlabeled GTP to the incubation medium (fig.1A, lane 2) but not by addition of 1 mM unlabeled ATP (not shown). In contrast to the single band labeled with [γ - 32 P]GTP, numerous proteins were labeled in the presence of [γ - 32 P]ATP (fig.1A, lane 3). In lane 4 it can be seen that addition of 1 mM unlabeled ATP abolished the labeling of these proteins.

Since [γ - 32 P]GTP is rapidly hydrolyzed by non-specific phosphohydrolase present in the membrane preparation it was necessary to find conditions that would help to maintain its concentration throughout the incubation period. In [5], mM levels of p(NH)ppA preserved low concentrations of GTP under similar conditions in experiments with rat liver and fat cell membrane preparations. Upon inclusion of 0.5 mM p(NH)ppA along with [γ - 32 P]GTP, we were surprised to find incorporation of label into numerous proteins (fig.1A, lane 5), many of which were similar or identical to those labeled with [γ - 32 P]ATP (cf. lane 3). This finding suggested the possibility of contamination of p(NH)ppA with precursors of ATP which, by a phosphotransferase reaction with [γ - 32 P]GTP, would give rise to [γ - 32 P]ATP and, consequently, to the labeling pattern seen in lane 5. This was tested by adding hexokinase and glucose as a means of removing any contaminating ATP present or formed in the incu-

bation medium. As shown in fig.1A, lane 6, this procedure eliminated the label in all bands except band a. Addition of hexokinase and glucose with [γ - 32 P]GTP alone did not prevent incorporation of label into band a (cf. lanes, 1,7). In contrast, inclusion of hexokinase and glucose in the test system containing [γ - 32 P]ATP (lane 3) led to complete elimination of all labeled protein bands (not shown).

The complications observed with p(NH)ppA lead

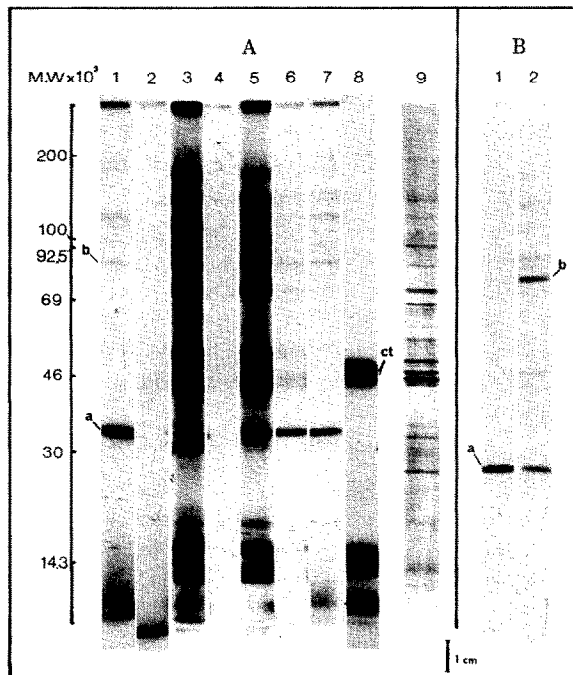


Fig.1. Separation of ovarian plasma membrane proteins by SDS-PAGE following incubation with [γ - 32 P]GTP or [γ - 32 P]ATP. (A) Purified rat ovarian plasma membranes (100 μ g protein) were incubated with [γ - 32 P]GTP, 0.5 μ M (40×10^6 cpm/assay) in the absence (lane 1) or presence (lane 2) of 1 mM unlabeled GTP as in section 2. In lanes 3 and 4 [γ - 32 P]ATP, 0.5 μ M (40×10^6 cpm/assay) substituted for [γ - 32 P]GTP and incubation was performed in the absence (lane 3) or presence (lane 4) of 1 mM unlabeled ATP. Plasma membranes were also incubated with [γ - 32 P]GTP and 0.5 mM p(NH)ppA in the absence (lane 5) or presence (lane 6), of hexokinase 50 U/ml and glucose 100 mM. Lane 7 describes the results of an experiment carried out as in lane 1 to which hexokinase and glucose at the above concentrations were added. Lane 8 contains a Triton X-100 extract of [32 P]ADP-ribosylated protein doublet prepared as in section 2. Lanes 1–7 and 9 contain 30 μ g protein each and Coomassie staining of a representative lane is given in lane 9. (B) Ovarian plasma membranes (130 μ g protein) were incubated with 1.2 μ M [γ - 32 P]GTP (3.7×10^7 cpm/assay) in the absence (lane 1) or presence (lane 2) of 1 mM CTP.

us to seek other means of retarding [γ - 32 P]GTP hydrolysis while at the same time minimizing internucleotide phosphotransfer. It was found that the inclusion of 1 mM CTP had no significant effect on the labeling of band 'a' by [γ - 32 P]GTP. However, CTP enhanced the labeling of an additional protein, designated 'b' (fig.1B, lane 2), of M_r 90 000 \pm 1000 (SEM, $n = 9$). This protein, can be seen also in fig.1A, lane 3 but, was only weakly labeled in the absence of CTP or p(NH)ppA and thus, not easily distinguished from background labeling. Direct determination of [γ - 32 P]GTP by chromatography on PEI-cellulose [5] revealed that also in the presence of CTP, degradation of the radiolabeled nucleotide was effectively inhibited. Addition of creatine phosphate and creatine kinase as a means of inhibiting internucleotide phosphotransfer was proposed [6] in studies on the catecholamine-sensitive GTPase in turkey erythrocytes. This condition was also found to be effective here with elimination of all the labeled bands present in fig.1A, lane 5 except for band a. However, the labeling of band a was reduced, probably as a result of lowering the specific radioactivity of [γ - 32 P]GTP upon regeneration of GDP.

The above findings support the view that ovarian plasma membranes contain activities which result in direct labeling of 2 major proteins a and b and which use GTP preferentially as a substrate. These results also emphasize the advantages in use of hexokinase and glucose as a means of selective discrimination of phosphotransfer from ATP to protein.

Cholera toxin catalyzes the ADP-ribosylation of proteins thought to be involved in the regulation of adenylate cyclase by GTP [15–17]. Fig.1A, lane 8, shows that incubation of ovarian plasma membranes with cholera toxin and [32 P]NAD leads to the labeling of 2 bands (lane 8)⁺ having app. M_r 44 000 \pm 2000 and 49 000 \pm 2000 (SEM, $n = 5$)*, a labeling pattern similar to that reported for membranes from other types of cells [15,24]. It is clear that the proteins labeled by the toxin are different from bands a or b. Furthermore, we concluded that under the

condition employed these GTP-binding regulatory proteins are not labeled by [γ - 32 P]GTP. However, these data do not exclude the possibility that the GTP-regulatory protein [7] labeled by cholera toxin and NAD may utilize GTP as substrate in a phosphotransferase reaction and that phosphorylation of bands a or b may be the result of such a reaction.

Ovarian plasma membranes labeled with [γ - 32 P]-GTP and the labeled protein bands punched out from slabs of SDS gels were subjected to enzymic proteolysis with *Staphylococcus aureus* protease [25]. SDS-PAGE of the labeled reaction products on 15–20% acrylamide gradients revealed that bands a and b are susceptible to proteolytic digestion, hence indicating their protein nature.

To test whether the proteins labeled with GTP were 'intrinsic' to the structure of the ovarian plasma membrane, we treated the membranes with various detergents after incubation with labeled [γ - 32 P]GTP in the presence of 1 mM CTP. The detergent-extracted soluble material(s) was separated from the insoluble membrane residue (p) by centrifugation and the pattern of the labeled proteins examined on SDS-PAGE. As seen in fig.2, band 'a' remained in the pellet after extraction of membranes with a variety of detergents. By contrast, band 'b' was extracted readily by Triton X-100 (lane 1s) and *n*-octylglucoside (lane 3s) but only partially by cholic acid (lane 2s). Note that a third band, designated 'c', became visible in all pellets, apparently due to extraction of all neighboring proteins. Its app. M_r is 56 000 \pm 200 (SEM, $n = 3$).

The results of the above experiments indicate that bands a and b differ in their structural relationship to the plasma membrane in that the former is not readily extractable with detergents whereas the latter is. This difference raises the possibility that band a is not an intrinsic membrane protein and may be of cytoskeletal origin. It has been reported that such proteins are not readily dissolved by various detergents unless high salt concentrations are included in the extraction mixture [26–28].

We next examined whether bands a and b are specific for rat ovarian plasma membranes. Using identical procedures described for labeling bands a and b by GTP in rat ovarian membranes, it was found that plasma membranes from the rat parotid gland contained band a and band b while the human erythrocyte ghost lacked band a (fig.3). In preliminary experiments with 'wild-type' S49 murine lymphoma cell plasma membranes, it was found that bands a and b were

⁺ Lane 8, fig.1 A also contains 2 additional more diffuse bands at $M_r < 14$ 300. These bands seem to originate from the cholera toxin preparation as indicated by a control experiment without ovarian plasma membranes (not shown)

* The isoelectric pH of the toxin-labeled proteins was found to be 6.0 and 6.5 for the M_r 44 000 and 49 000 species, respectively

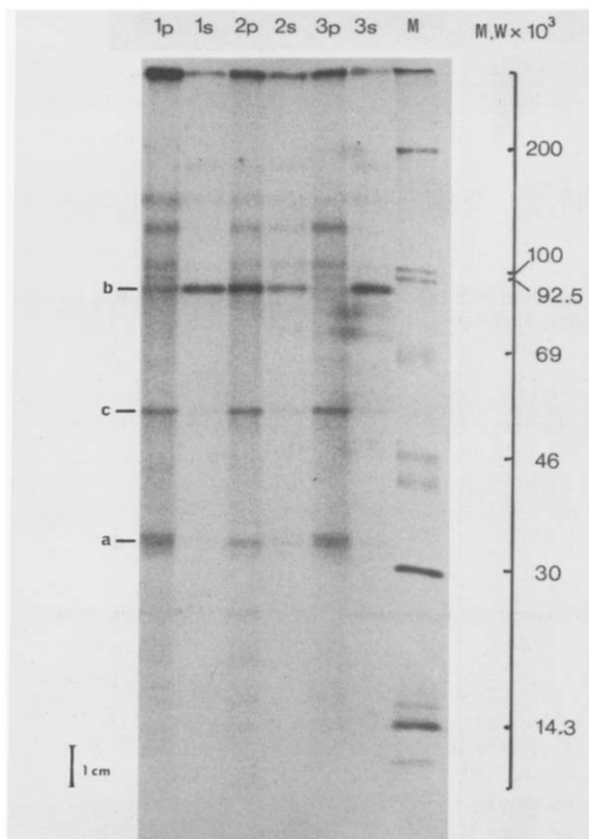


Fig.2. The effect of treatment of the ^{32}P -labeled plasma membranes by various detergents. Plasma membranes (130 μg protein) were incubated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, 1.2 μM , (3.7×10^7 cpm/assay) in the presence of 1 mM CTP as in section 2. The washed membrane pellets were extracted with a detergent solution for 1 h at 4°C and subsequently centrifuged for 5 min at $100\,000 \times g$ (airfuge; Beckman). The resulting supernatants (s) and pellets (p) were prepared for SDS-PAGE as in section 2. The extraction solution contained 0.7% Triton X-100 (lanes 1(s), 1(p)), 1.0% cholic acid (lanes 2(s), 2(p)) and 1% *n*-octyl glucoside (lanes 3(s) and 3(p)). Lane 'M' contains the ^{14}C -methylated marker proteins as in section 2.

labeled with GTP. Extracting parotid membranes with detergents as in fig.3 for ovarian membranes, indicated that bands a and b in the 2 tissues behave identically also with respect to their solubility in detergents.

This study suggests that some plasma membrane proteins may be directly phosphorylated by enzymes that use GTP as preferred substrate. The nature of the enzymes involved, as well as the acceptor proteins or the possibility that bands a and/or b represent GTP-requiring enzyme-intermediates must await further studies.

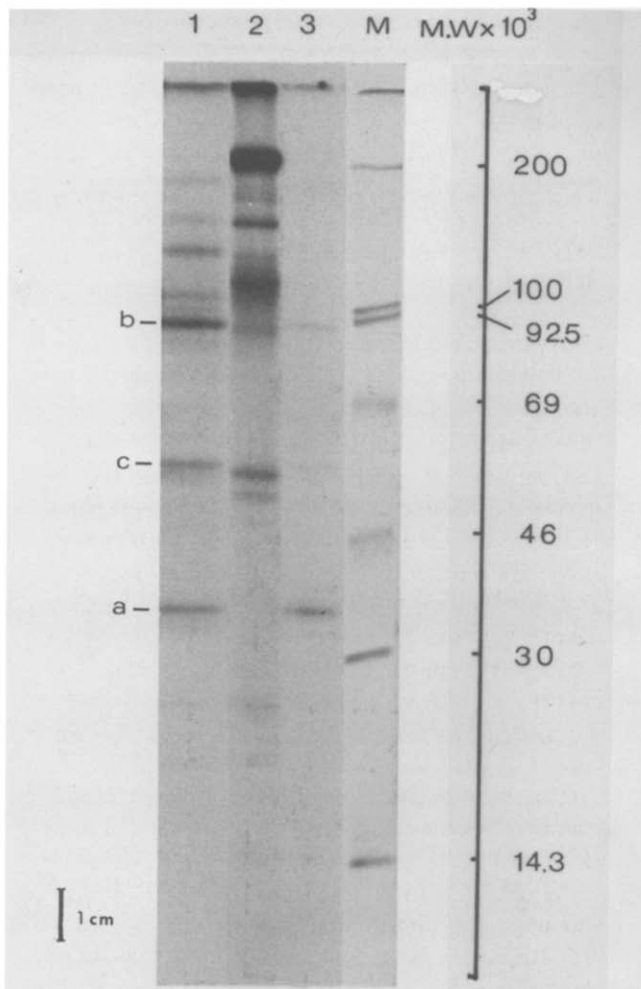


Fig.3. Comparison of a GTP-dependent labeling profile of plasma membrane preparations derived from rat parotid gland, rat ovary and of human erythrocyte ghosts. Ovarian plasma membranes (120 μg protein) (lane 1), human erythrocyte ghosts (300 μg protein) (lane 2) and parotid membranes (300 μg protein) (lane 3) were incubated with 1.2 μM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (4×10^7 cpm) and prepared for SDS-PAGE as in section 2. Lane 'M' contains the ^{14}C -methylated marker proteins.

It would also appear that the same labeled proteins seen in ovarian membranes are present in other cell membranes, notably parotid and S49 lymphoma cells, but not in the membrane of the human erythrocyte ghost. It is of interest that the human erythrocyte membrane lacks adenylate cyclase but contains the GTP-regulatory protein which is acted upon by cholera toxin and NAD [24]. A 100 000 M_r protein which is labeled by $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ has recently been described in

plasma membranes of mouse fibroblasts [29] and may correspond to band b reported here. Whether the phosphorylated proteins reported here have any bearing on the ability of GTP to activate adenylate cyclase or to contribute to the process of hormone-induced desensitization [12] remains to be seen.

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