# α-Subunits of $N_s$ are released from the plasma membrane following cholera toxin activation

Christopher J. Lynch, Laura Morbach, Peter F. Blackmore+ and John H. Exton*

*Laboratories for the Studies of Metabolic Disorders, Howard Hughes Medical Institute and the Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232, USA*

Received 3 March 1986

Cholera toxin (CT) and islet-activating protein (IAP, a *Bordetella pertussis* toxin) were employed to test the hypothesis that GTP-binding regulatory proteins are released from plasma membranes to a greater extent when 'activated' than when 'inactivated'. CT, which activates $N_s$ (the stimulatory GTP-binding regulatory protein of the adenylate cyclase system), catalyzed the incorporation of radioactivity from [$^{32}$P]NAD into 45 and 47.5 kDa peptides associated with rat liver plasma membranes. Following ADP-ribosylation and centrifugation at 100000 × *g* for 1 h, approx. 30–35% of these CT-labelled peptides were no longer associated with the plasma membranes, but were recovered from the supernatant fraction. IAP, which inactivates $N_i$ (the inhibitory GTP-binding regulatory protein of the adenylate cyclase system) catalyzed the incorporation of radioactivity from [$^{32}$P]NAD into a 41 kDa peptide associated with the membranes. However, in contrast to the CT-labelled peptides, typically less than 5% of the IAP-labelled peptide was found in the 100000 × *g* supernatant fraction, but rather was almost exclusively associated with the membrane pellet. The data indicate that the α-subunits of $N_s$ are released from the plasma membrane following activation, and support the hypothesis that the βγ-subunits act to anchor the α-subunits to the plasma membrane.

*Cholera toxin* *Islet-activating protein* *GTP-binding protein*

## 1. INTRODUCTION

Rodbell and co-workers [1–3] have provided some preliminary evidence for a theory which proposes that, as a consequence of hormone stimulation or exposure to agents which activate GTP-binding proteins (e.g. fluoride, Mg, GTP, etc.), the α-subunits of these proteins are released from the plasma membrane. It is further proposed that the released α-subunits may then be free to interact with other components of the cell and/or plasma membrane. Thus the β- and/or γ-subunits of these proteins would behave as moorings for attachment of the α-subunits to the plasma membrane. Recent evidence from Sternweis [4] showing that α-subunits of GTP-binding regulatory proteins cannot associate with plasma membranes in the absence of βγ-subunits, would seem to support Rodbell's hypothesis.

In this study, cholera toxin (CT) and islet-activating protein (IAP) were employed to label and activate or inactivate, respectively, $N_s$ and $N_i$ in purified rat liver plasma membranes. It is shown that as a consequence of the action of CT, two [$^{32}$P]ADP-ribosylated peptides are released from the plasma membrane and these released peptides represent approx. 30–35% of the total 45 and 47.5 kDa labelling. In contrast, less than 5% of the 41 kDa peptides [$^{32}$P]ADP ribosylated by IAP were released from the plasma membrane. These data support the theory that release of α-subunits from plasma membranes may be a consequence of the activation of a GTP-binding regulatory protein.

## 2. MATERIALS AND METHODS

[$^{32}$P]NAD was obtained from New England

+ Associate Investigator, Howard Hughes Medical Institute
* Investigator, Howard Hughes Medical Institute

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/86/$3.50 

Nuclear (Boston, MA). IAP was purchased from List Biological Laboratories (Campbell, CA) and was activated as described [5]. CT A subunit was obtained from either List Biological Laboratories or Calbiochem (La Jolla, CA). $M_r$ standards were purchased from BioRad (Richmond, CA). Sources of other materials have been described [5,6].

Plasma membranes isolated from a rat liver by density gradient centrifugation [6] using Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) were divided equally into two centrifuge tubes and washed in ice-cold isolation media containing isotonic sucrose, 5 mM Na-Hepes (pH 7.4), 1 mM EGTA and 20 $\mu$g/ml each of leupeptin and antipain. One pellet (approx. 5–6 mg of protein) was then resuspended in 1.5 ml of IAP-ribosylating buffer containing 1 mM EDTA (pH 7.4), 10 mM thymidine and protease inhibitors (20 $\mu$g/ml each of leupeptin and antipain), while the other pellet was resuspended in CT-ribosylating buffer containing 400 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, 10 mM thymidine and protease inhibitors as above. Conditions for toxin-catalyzed ADP-ribosylation were individualized for each toxin to optimize labelling as recommended by Riberio-Neto et al. [7]. The IAP-stimulated [$^{32}$P]ADP-ribosylation assay mixture contained final concentrations of the following in 500 $\mu$l: 10 mM [$^{32}$P]NAD (50–60 $\mu$Ci/tube), 50 mM Na-Hepes (pH 7.4), 10 mM thymidine, 1 mM EDTA, 1 mM ATP, 0.1 mM GTP, 50 $\mu$g/ml of activated IAP, 400 $\mu$l of membranes in IAP-ribosylating buffer (see above) and protease inhibitors as above. The CT [$^{32}$P]ADP-ribosylation assay mixture contained final concentrations of the following in 500 $\mu$l: 10 mM [$^{32}$P]NAD (50–60 $\mu$Ci/tube), 50 mM Na-Hepes (pH 7.4), 400 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 10 mM thymidine, 1 mM EGTA, 1 mM ATP, 0.1 mM GTP, 50 or 100 $\mu$g/ml of CT A subunit, 400 $\mu$l of membranes in CT-ribosylating buffer (see above) and protease inhibitors as above. Both assays were carried out at 30°C and were started with the addition of [$^{32}$P]NAD, ATP and GTP. Additional [$^{32}$P]NAD, ATP and GTP were added at 7 min intervals for 27 min.

Following this period, the mixtures were differentially adjusted as necessary to yield final concentrations of the following in 1 ml: 25 mM Na-Hepes (pH 7.4), 200 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM thymidine, 20 $\mu$g/ml each of leupeptin and antipain. After an additional 10 min with shaking at 30°C, 50 $\mu$l fractions (for determination of total labelling) of each tube were precipitated in an Eppendorf tube containing 1 ml of 15% trichloroacetic acid, 45% EtOH. A volume (400 $\mu$l) of the remainder was transferred to a centrifuge tube and centrifuged for 1 h at 100000 × $g$ (4°C) in a Beckman airfuge fitted with an A-95 rotor. 50 $\mu$l of each supernatant was then precipitated with trichloroacetic acid as described above for determination of soluble labelled proteins, and the remaining supernatant was carefully discarded. The 100000 × $g$ pellets were resuspended in a 400 $\mu$l final volume of isolation media and 50 $\mu$l of this mixture was also precipitated with trichloroacetic acid for the determination of membrane associated ADP-ribosylated proteins.

Precipitated proteins were sedimented at 11000 × $g$ for 5 min and the supernatant was removed. The protein was then solubilized by boiling in 300 $\mu$l of 12% SDS, 25% glycerol, 25 mM DTT, 20 mM Tris, 0.15 M glycine buffer (pH 8.2) and 0.03% bromophenol prior to SDS-PAGE of 50 or 100 $\mu$l of each sample in duplicate. $M_r$ standards were labelled for autoradiography with EMIT (New England Nuclear) following Coomassie blue staining. Autoradiographs were prepared from the dried gels by overnight exposure (−70°C) of Kodak XAR-5 film in cassettes with Dupont Quanta III enhancing screens. The radioactive contents of the 45–48 kDa ($N_s\alpha$) and 41 kDa ($N_i\alpha$) regions of the gels were determined by slicing these $M_r$ regions from wet gels into vials containing 10 ml Beckman Redisolv EP and counting in a Beckman LS 1800 liquid scintillation counter. The experiments shown are representative of three such studies.

## 3. RESULTS

Fig.1 shows that a number of major proteins isolated with liver plasma membranes are partly lost during centrifugation following exposure at 30°C to constituents of the ADP-ribosylation assay mixtures. Neither CT-catalyzed ADP-ribosylation nor IAP-catalyzed ADP-ribosylation differentially affected the release of these major

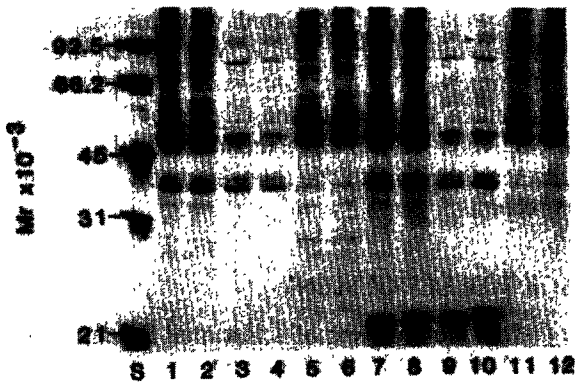


Fig.1. Coomassie blue-stained SDS-polyacrylamide gel showing the release of soluble proteins from isolated liver plasma membranes. IAP- (lanes 1–6) and CT- (lanes 7–12) catalyzed [$^{32}$P]ADP-ribosylation of isolated liver plasma membranes was performed under different conditions as described in section 2. Membranes were then incubated under similar conditions prior to centrifugation and SDS-PAGE of proteins from total membranes (lanes 1,2,7,8), 100000 × *g* supernatant fraction (lanes 3,4,9,10) and resuspended 100000 × *g* pellet (lanes 5,6,11,12). Lane S contains Coomassie blue-stained (EMIT-labelled) $M_r$ standards. The peptides in the 21–31 kDa region of the gels which appear in lanes 1–6 but not lanes 7–12 (and vice versa) are subunits of the toxins which are also precipitated by trichloroacetic acid.

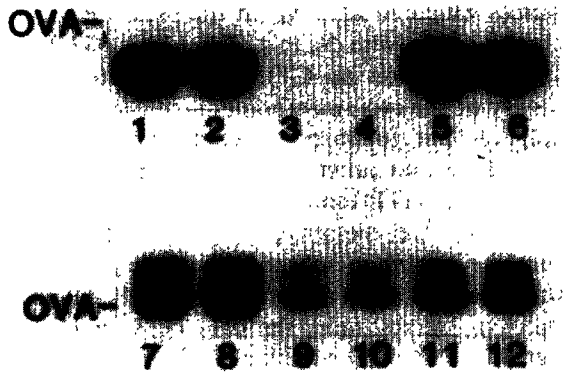


Fig.2. Release of [$^{32}$P]ADP-ribosylated proteins from liver plasma membranes. Two portions of an autoradiograph obtained from the gel in fig.1 showing [$^{32}$P]ADP-labelling of peptides by IAP (lanes 1–6) and CT (lanes 7–12). OVA, position of ovalbumin relative to the labelled peptides.

proteins as determined from Coomassie blue staining.

CT catalyzed the incorporation of radioactivity from [$^{32}$P]NAD into two peptides of approx. 45 and 47.5 kDa. Following incubation in a medium containing a mixture of the CT and IAP-assay components and then centrifugation, approx. 30–35% of these two CT-labelled peptides were found in the 100000 × *g* supernatant (fig.2). In contrast, typically less than 5% of the 41 kDa peptide labelled in the presence of IAP was apparent in 100000 × *g* supernatant fraction. The figure only shows the region of gels in the vicinity of the 43 kDa ovalbumin marker protein, but, as shown in previous reports from our laboratory [5,8], the toxins do not significantly ADP-ribosylate other proteins in liver plasma membranes.

## 4. DISCUSSION

$N_s$ and $N_i$ are part of a family of homologous GTP-binding regulatory proteins that act as signal transducers and amplifiers in the adenylate cyclase system. These two proteins are thought to be heterotrimeric, consisting of $\alpha$-, $\beta$- and $\gamma$-subunits (review see [9]). The $\beta\gamma$-subunits for each protein are thought to be very similar or identical, while the $\alpha$-subunits differ in $M_r$ and in activity in the adenylate cyclase system. While the specific activity of these proteins is typically enriched in plasma membrane fractions, three studies have emerged which suggest that the $\alpha$-subunits of these proteins may not be strictly limited to this structure [1,10–12]. For instance, Pecker and Hanoune [10] demonstrated that a nucleotide-containing protease-sensitive factor is present in rat liver cytosol fractions which enhances GTP regulation of epinephrine-sensitive adenylate cyclase activity (this is presumed to be, but not positively identified as, the $\alpha$-subunit of $N_s$). Similarly Bhat and co-workers [11] have isolated supernatant fractions from a number of tissues which confer guanine nucleotide and fluoride sensitivity on the adenylate cyclase of cyc$^-$ mutants of S49 murine lymphoma cells. Nakamura and Ui [12] reported the predominant occurrence of the $\alpha$-subunit of $N_i$ (or a similar IAP-sensitive protein) in the cytosol fraction of mast cells.

Neilsen et al. [1] have shown that when plasma membranes from cyc$^-$ mutants of S49 cells are incubated with plasma membranes from other

sources which contain $N_s$, in the presence of factors which activate $N_s$ (e.g. $F^-$, $Mg^{2+}$, GTP and ADP-ribosylation by CT), the adenylate cyclase activity of the $cyc^-$ membranes increases. Rodbell [2,3] has put forward a mechanistic hypothesis from these and other data which depicts the $\alpha$-subunits of GTP-binding regulatory proteins as 'programmable messengers' which are released from the plasma membrane when exposed to activating agents (such as hormones, F, Mg and GTP analogs). Interestingly, the data of Sternweis [4] suggest that $\beta\gamma$-subunits of GTP-binding regulatory proteins may indeed act to anchor $\alpha$-subunits to the plasma membranes.

In this study we have tested the hypothesis that inactivation of a GTP-binding protein may prevent its dissociation from the plasma membrane, while activation may promote its release. $N_s$ was activated by incubation of membranes with [$^{32}$P]NAD and CT, while $N_i$ was inactivated by incubation with [$^{32}$P]NAD and IAP. [$^{32}$P]ADP-ribosylation by CT resulted in the labelling of 45 and 47.5 kDa peptides (which are presumably associated with the activation of the adenylate cyclase system), whereas IAP [$^{32}$P]ADP-ribosylated a single 41 kDa peptide (presumably the $\alpha$-subunit of $N_i$) (see [5] for references). Following incubation in a common medium and centrifugation at $100000 \times g$, a number of major proteins were found in the supernatant fractions as detected by Coomassie blue staining (fig.1). The toxins appeared to have no differential effect on the release of these major proteins from liver membranes. However, the toxins did appear to have a differential effect on the release of [$^{32}$P]ADP-ribosylated peptides. Fig.2 shows that a significant percentage of the two peptides labelled by [$^{32}$P]NAD and CT was no longer associated with the plasma membrane following centrifugation, while the 41 kDa peptide labelled by IAP remained almost exclusively associated with plasma membrane. While further studies will be required to determine whether hormones such as glucagon and angiotensin II promote the release of $N_s$ and $N_i$, respectively, from plasma membranes, the present data lend support to the programmable messenger hypothesis.

## ACKNOWLEDGEMENT

C.J.L. is a recipient of a National Research Service Award (AM 07274) from the US Public Health Service.

## REFERENCES

[1] Nielsen, T.B., Lad, P.M., Preston, M.S. and Rodbell, M. (1980) Biochim. Biophys. Acta 629, 143–155.
[2] Rodbell, M. (1985) Trends Biochem. Sci. 119, 461–464.
[3] Rodbell, M. (1985) in: Pertussis Toxin (Sekura, R.D. et al. eds) pp.65–76, Academic Press.
[4] Sternweis, P.C. (1986) J. Biol. Chem. 261, 631–637.
[5] Lynch, C.J., Prpic, V., Blackmore, P.F. and Exton, J.H. (1986) Mol. Pharmacol. 29, 196–203.
[6] Prpic, V., Green, K.C., Blackmore, P.F. and Exton, J.H. (1984) J. Biol. Chem. 259, 1382–1385.
[7] Ribiero-Neto, F.A.P., Matteca, R., Hildebrant, J.D., Codina, J., Field, J.B., Birnbaumer, L. and Sekura, R.D. (1985) Methods Enzymol. 109, 566–572.
[8] Uhing, R.J., Prpic, V., Jiang, H. and Exton, J.H. (1986) J. Biol. Chem. 261, 2140–2146.
[9] Gilman, A.G. (1984) Cell 36, 577–579.
[10] Pecker, F. and Hanoune, J. (1977) J. Biol. Chem. 252, 2784–2786.
[11] Bhat, M.K., Iyengas, R., Abramowitz, J., Bordelon-Riser, M.E. and Birnbaumer, L. (1980) Proc. Natl. Acad. Sci. USA 77, 3836–3840.
[12] Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584–3593.