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CROSS-LINKING OF G-PROTEINS TO THE PROLACTIN RECEPTOR IN RAT NB2 LYMPHOMA CELLS

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SUMMARY. In Nb2 cell membranes, two guanine nucleotide-binding protein (G-protein) species (Mr 43.5 and 46.5 kD) were [^{32}P]-ADP-ribosylated by cholera toxin, while a single protein (Mr 41.5 kD) was [^{32}P]-ADP-ribosylated by pertussis toxin. Immunostaining indicated two immunoreactive prolactin (PRL) receptor moieties of 56 and 64 kD. Cross-linking with ethylene glyco bis{succinimidyl-succinate} (mol. length of 16.1 Å) generated a high mol. wt., [^{32}P]-ADP-ribosylated band of 140-160 kD which also showed immunoreactivity with antiserum to the PRL receptor. Other cross-linkers with shorter molecular lengths (8.6 - 11.4 Å) were ineffective. These findings indicate that the Nb2 lactogen receptor is complexed with G-proteins and provide evidence for the role of G-proteins in mediating PRL-stimulated mitognesis in Nb2 cells.

G-proteins have been implicated in the mechanism of signal transduction for PRL- and interleukin-2-stimulated mitogenesis in the lactogen-dependent rat Nb2 lymphoma cells (1,2). The α-subunits of several G-protein species are susceptible to ADP-ribosylation by cholera (eg. Gs and Gt) and pertussis toxins (eg. Gi, Gt and Go). In the presence of these bacterial toxins, [^{32}P]-ADP-ribosylated G-proteins have been visualized in the Nb2 cell membranes (2,3). Both toxins also inhibited PRL-stimulated DNA synthesis and proliferation of Nb2 cells (2). Furthermore, guanine nucleotides inhibited the binding of [^{125}I]-human growth hormone to the lactogen receptor, an effect that is characteristic of receptors coupled to G-proteins (2).

The present study further examined the role of the G-proteins in the signal transduction pathway of the PRL receptor in Nb2 cells. The proximity of the PRL receptor to G-proteins was investigated.

MATERIALS AND METHODS

Ovine PRL (NIH-P-S-12) was a gift from NIAMDD, Bethesda, MD. Guinea pig antisera to rabbit mammary gland PRL receptors (4) and rabbit antisera to guinea pig IgG were raised in this laboratory. The other materials and their sources were:

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Protoblot alkaline phosphatase immunostaining (rabbit) kit (Promega, Bio/Can Scientific Inc, Mississauga, Ontario, Canada); adenylate-[^{32}P]-nicotinamide adenine dinucleotide (NEN Research Products, Boston, MA); cholera and pertussis toxins (List Biological Laboratories, Inc., Campbell, CA); ATP and GTP (Sigma Chemical Co., St. Louis, MO); cross-linkers EGS (ethylene glyco bis{succinimidyl-succinate}), DMA (dimethyl adipimidate.2HCl), DMS (dimethyl suberimidate.2HCl) and DSS (disuccinimidyl suberate) (Pierce Chemical Co., Rockford, IL); mol. wt. markers (Biorad, Mississauga, Ontario, Canada); Fischer's medium for leukemic cells and fetal bovine serum (FBS) (Grand Island Biological Co. Canada, Burlington, Ontario, Canada); lactogen-free horse serum (HS) (Flow Co., McLean, VA). All other chemicals were of analytical grade.

Membrane preparation: Suspension cultures of Nb2 cells (maintained in Fischer's medium-10% FCS-10% HS) and stationary cultures (obtained by incubating confluent cells overnight in Fischer's medium-10% HS) were used as previously described (5). Cell membranes from stationary Nb2 cells (1 x 10^6 cells/ml) were prepared and ADP-ribosylation of Nb2 membrane proteins was carried out as previously described (2,3).

Cross-linking of membrane proteins: This was as described by Hughes et al. (1983) (6). Briefly, EGS, DMA, DMS or DSS (25 mM in dimethylsulfoxide) was added to the various membrane samples at a final concentration of 0.25 mM. After 30 min at 21°C, 0.1 vol of 1M Tris-HCl, pH 7.6 was added and the samples were centrifuged for 10 min in a microfuge. The cell membrane pellets were washed twice in 10 mM potassium phosphate buffer, pH 7.5.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting: The radiolabeled and/or cross-linked membranes were dissolved in 1% SDS-5% β-mercaptoethanol-10% glycerol-62.5 mM Tris-HCl, pH 7.5-0.02% bromophenol blue and heated for 3 min at 100°C. The samples were electrophoresed at 200V in 0.75 mm thick SDS-polyacrylamide (10 - 15%) gels using a mini Protean gel apparatus (Bio-rad Laboratories Canada Ltd., Mississauga, Ontario, Canada). The gels were then soaked in 25 mM Tris-HCl, pH 8.3-192 mM glycine-20% methanol for 30 min. Transfer onto nitrocellulose paper was carried out at 300 mA for 3 h at 10°C. The blots were blocked with TBS-3% BSA (10 mM Tris-HCl, pH 8.0-150 mM NaCl - 3% blot qualified bovine serum albumin) for 30 min at room temperature and then incubated with the first antibody, guinea pig anti-PRL receptor (1:500) in TBST (TBS-0.05% Tween-20)-1% BSA, for 1 h at room temperature. Incubation with the second antibody, rabbit anti-guinea pig IgG (1:100) in TBST-1% BSA, was for 1 h and with the third antibody, anti-rabbit-alkaline phosphatase conjugate (1:7500) in TBST-1% BSA, was for 30 min. Each incubation was followed by three 5 min washes in TBST. Color development was carried out with the alkaline phosphatase substrates BCIP and NST (Promega) in 100 mM Tris-HCl, pH 9.5-100 mM NaCl- 5 mM MgCl2. The blots were wrapped in clear plastic for autoradiography at -70°C for 24 h using Kodak X-Omat film (Eastman Kodak, Rochester, NY).

RESULTS

[^{32}P]-ADP-ribosylation of Nb2 membrane proteins

Two G-protein species (Mr 43.5 and 46.5 kD) were ADP-ribosylated by cholera toxin, while a single protein (Mr 41.5 kD) was ADP-ribosylated by pertussis toxin (Fig. 1). These bands were absent from controls which received no toxin treatment. A [^{32}P]-labeled band of more than 92.5 kD was consistently obtained in controls and toxin-treated samples (Fig. 1).

Cross-linking of Nb2 PRL receptor to G-proteins

In order to determine the proximity of the Nb2 PRL receptor to the Nb2 G-proteins, G-proteins in the Nb2 cell membranes were first [^{32}P]-ADP-ribosylated in

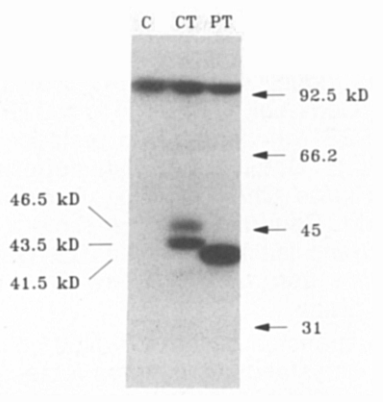

Fig. 1. [^{32}P]-ADP-ribosylation of Nb2 membrane proteins. ADP-ribosylation was carried out either in the presence of cholera or pertussis toxin as described in Materials and Methods. Controls (C) received no toxin treatment.

the presence of either toxin. Cross-linking reactions were then carried out with one of several homobifunctional cross-linkers. The [^{32}P]-ADP-ribosylated and cross-linked membrane samples were subjected to SDS-PAGE and transferred onto nitrocellulose. The blots were analyzed by immunostaining with antiserum to the PRL receptor followed by autoradiography to detect [^{32}P]-ADP-ribosylated bands.

Autoradiography of the electrophoresed [^{32}P]-ADP-ribosylated Nb2 membrane samples in the presence of cholera or pertussis toxin revealed the toxin-sensitive G-proteins (Fig.2A). Immunostaining of the same blot indicated two bands (Mr 56 and 64 kD) showing immunoreactivity with guinea pig antiserum to the PRL receptor (Fig. 2B). No immunoreactive band was obtained with normal guinea pig serum (data not shown).

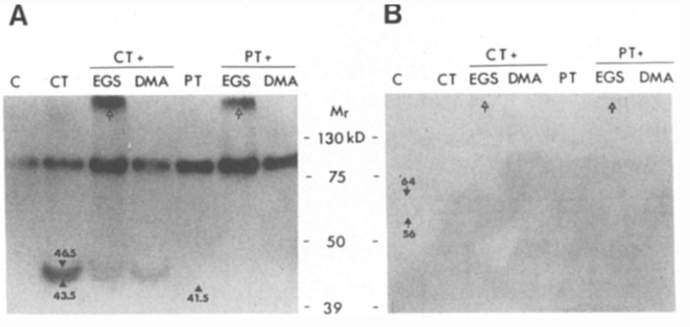

Fig. 2. Cross-linking of Nb2 PRL receptor and G-proteins. (A) Nb2 cell membranes, prepared as described in Materials and Methods, were [^{32}P]-ADP-ribosylated in the absence (lane C) or presence of cholera or pertussis toxin. Cross-linking of toxin-treated samples were carried out with the addition of the cross-linker EGS or DMA. The samples were electrophoresed in a 15% SDS-polyacrylamide gel, transferred onto nitrocellulose paper and autoradiographed at -70°C. (B) The blot corresponding to the autoradiogram in (A) was immunostained with antiserum to PRL receptor. Mol. wt. markers are indicated on the side. Open arrows indicate [^{32}P]-ADP-ribosylated and immunoreactive bands.

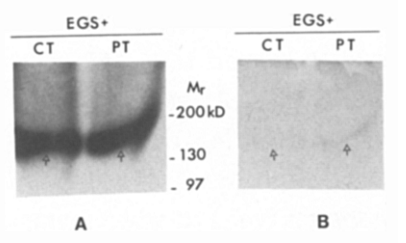

Fig. 3. Cross-linking of Nb2 PRL receptor and G-proteins : 10 % gel. [^{32}P]-ADP ribosylation, cross-linking with EGS and Western blotting were carried out as in Fig 2. The samples were electrophoresed in a 10% SDS-polyacrylamide gel. (A) [^{32}P]-labeled G-proteins. (B) Western blotting with antiserum to PRL receptor. Mol. wt. markers are indicated on the sides. Open arrows indicate [^{32}P]-ADP-ribosylated and immunoreactive bands.

Cross-linking with EGS (mol. length of 16.1 Å), after [^{32}P]-ADP-ribosylation, resulted in a [^{32}P]-ADP-ribosylated band of high mol. wt. which barely entered the 15% resolving gel (Fig. 2A; open arrow). This band was also detected by Western blotting with anti-PRL receptor (Fig. 2B; open arrow). However, cross-linkers with shorter molecular lengths (DMA, 8.6 Å; DMS, 11 Å; DSS, 11.4 Å) were without effect (Fig. 2B and data not shown).

The EGS-linked high mol. wt. band was further resolved on a 10% SDS-polyacrylamide gel (Fig. 3). A [^{32}P]-ADP-ribosylated band, Mr 140-160 kD, was obtained (Fig. 3A) which was immunoreactive with antiserum to the PRL receptor (Fig. 3B).

DISCUSSION

The present report provides direct evidence for the association of the lactogen receptor with G-proteins. In the presence of the cross-linker EGS (spatial distance of 16.1 Å), a high mol. wt. band of 140 - 160 kD was obtained which was both [^{32}P]-ADP-ribosylated by cholera and pertussis toxins and showed immunoreactivity with antiserum to the PRL receptor. We have previously demonstrated the presence of cholera and pertussis toxin-sensitive G-proteins in the Nb2 cells which were shown to express mRNA transcripts for Gs, Gi2 and Go (2,3). The binding of [^{125}I]-human growth hormone to the lactogen receptor in Nb2 cells was decreased in a dose-dependent manner by the guanine nucleotides GTP, GDP and the analog guanosine 5'-0-(3-thiotriphosphate), indicating that the lactogen receptors are coupled to G-proteins (2).

Previous reports have indicated mol. wt.s of 41.5 and 64.9 kD (7) or 42, 52 and 64 kD (8) for the Nb2 PRL receptors. In the present study, Western blotting of Nb2 membrane proteins with guinea pig antiserum to the PRL receptor revealed two immunoreactive bands of mol. wt. of 56 and 64 kD. Since the cholera toxin substrates are approximately 43.5 and 46.5 kD and the pertussis toxin substrate is approximately 41.5 kD, the coupling of either the 56 kD or the 64 kD species of the PRL receptor to both pertussis toxin (41.5 kD) and cholera toxin (either 46.5 or 43.5 kD) substrates

would result in bands ranging from 141 to 152 kD. These combinations would account for the immunoreactive and [^{32}P]-labeled 140 - 160 kD band obtained with EGS. Of the cross-linkers used, only EGS (16.1 Å) was effective in the cross-linking reactions. DMS (11 Å), DMA (8.6 Å) and DSS (11.4 Å) were without effect. EGS has also been used successfully to cross-link [^{125}I]-hGH with the lactogen receptor (6).

The PRL receptors in the rat liver and rabbit mammary gland have been cloned and sequenced (9,10). Structural analysis indicated that the cytoplasmic domain in the rabbit PRL receptor is much longer than that of the rat PRL receptor. Recently, two groups of investigators have each described short and long forms of the rat ovarian PRL receptor (11,12). In one case (11), the long form of the PRL receptor was found to contain consensus sequences for an ATP/GTP binding site. However, this observation has not been confirmed (12). It has been suggested that distinct types of PRL receptors, differing in the lengths of their cytoplasmic domain, may trigger different biological actions of PRL (10,11). The reported mol. wt.s of the Nb2 PRL receptors are different from that in other tissues (6-8) and Nb2 PRL receptors have considerably higher affinity for PRL than the receptors in normal target tissues (4). Thus, the mechanism of PRL signal transduction in the Nb2 cells and in other target tissues may be different and remains to be determined.

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