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GLYCOSYLATION-DEPENDENT BINDING OF PANCREATIC TYPE I PHOSPHOLIPASE A_2 TO ITS SPECIFIC RECEPTOR

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Summary: Pancreatic group I phospholipase A_2 (PLA_2-I) elicits various biological responses via its specific receptor. The PLA_2-I binding to its recombinant soluble receptor was considerably reduced after Peptide: N-glycosidase F treatment of the receptor. In cultured bovine smooth muscle cells, treatment with tunicamycin, a N-glycosylation inhibitor, resulted in a decrease in the number of PLA_2-I receptor. In addition, the PLA_2-I binding was blocked by the addition of a lectin, Wheat germ agglutinin. These results suggest an involvement of N-linked oligosaccharides of the PLA_2-I receptor for its ligand recognition. © 1995 Academic Press, Inc.

Mammalian secretary phospholipases A_2 are classified into two types, the pancreatic group I (PLA_2-I) and the arthritic group II (PLA_2-II), based on their primary structures (1). PLA_2-I has been thought to act as a digestive enzyme because of its abundance in digestive organs (2,3). However, our recent studies identified new physiological functions of this type of PLA_2 via its specific receptor (4); PLA_2-I is found to elicit DNA synthesis (5,6), contraction (7,8), chemokinetic cell migration (9), and eicosanoid production (10-12), as receptor-mediated responses. The PLA_2-I receptor has recently been purified from the membranes of bovine corpus luteum (13). Analysis of its cDNA sequence reveals that the PLA_2-I receptor consists of 8 tandem repeats homologous to C-type carbohydrate-recognition domains and a single transmembrane region followed by a short cytoplasmic tail (14). In addition, the receptor was identified as a glycoprotein which contains 15 potential N-glycosylation

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sites. In the present study, we examined a role of its carbohydrate moieties in the recognition of ligand using a recombinant soluble form of the bovine PLA_2-I receptor that lacks the transmembrane as well as cytoplasmic domains and retains a high affinity and specificity for PLA_2-I (14). We also examined the effect of tunicamycin, a known N-glycosylation inhibitor (15), on the PLA_2-I binding to the receptor in cultured smooth muscle cells isolated from bovine aorta (BSMC).

MATERIALS AND METHODS

Materials: Sodium [^{125}I]iodide (carrier-free, 3.7 GBq/ml) was purchased from Amersham Corp. Porcine [^{125}I]PLA_2-I (420-480 cpm/fmol) was prepared by using a chloramine T method as described in our previous paper (6). Tunicamycin, Concanavalin A and Wheat germ agglutinin were purchased from Sigma. Peanut lectin, Dolichos lectin and L-fucose were purchased from Seikagaku Corp. N-acetylneuraminic acid was obtained from Wako Pure Chemical Industries, Corp. D-mannose, N-acetyl-galactosamine, N-acetylglucosamine and D-galactose were purchased from Nacalai Tesque. Peptide: N-glycosidase F (PNGase F), isolated from *Flavobacterium meningosepticum*, was obtained from Boehringer-Mannheim Biochemica. Other chemicals were purchased from commercial sources.

Treatment of Recombinant Soluble PLA_2-I Receptor with PNGase F: Soluble PLA_2-I receptor, which lacks the transmembrane and cytoplasmic regions (14), was produced in CHO cells under the control of a strong eukaryotic promoter SRα (16) with an expression vector carrying dihydrofolic acid reductase gene, as described previously (17). Purification of the receptor was carried out using PLA_2-I-coupled affinity column (13). A soluble receptor (1 μg) was incubated with or without 1.2 U of PNGase F in 20 mM Na-phosphate (pH 7.5) containing 50 mM EDTA and 0.02 % NaN_3, for 18 hr at 37°C. Half of the samples was then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5 % gel) under the reducing conditions followed by silver staining. For binding assay, the PLA_2-I receptors (10 ng) were incubated with 1 nM [^{125}I]PLA_2-I for 2 hr at 4°C, and the binding activity was measured by the polyethylene glycol-precipitation method (6). Specific binding is defined as the differences between in the absence and presence of 500 nM unlabeled PLA_2-I.

Effects of Tunicamycin and Lectins on the [^{125}I]PLA_2-I Binding to Cultured Bovine Smooth Muscle Cells: Bovine smooth muscle cells (BSMC) were isolated from thoracic aorta as described previously (18). The cells were cultured in Dulbecco's modified minimum essential medium containing 10% horse serum, penicillin (100 unit/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air at 37°C. Treatment of BSMC with tunicamycin was carried out when the cells were approximately 75% confluent in 6-well plates. The [^{125}I]PLA_2-I binding activity was examined as described previously (6). The effects of a variety of lectins and/or monosaccharides were

examined by incubating them with BSMC for 15 min followed by the addition of [^{125}I]PLA_2-I (3 nM).

RESULTS AND DISCUSSION

Analysis of the cDNA sequence encoding the bovine PLA_2-I receptor reveals the presence of 15 potential N-linked glycosylation sites (14). The role of its carbohydrate moieties was first examined using a soluble form of PLA_2-I receptor purified from transfected CHO cells. Treatment of the receptor with PNGase F, which releases N-linked oligosaccharides from the glycoproteins (19), resulted in a reduction of its molecular weight from 190 kDa to ca. 150 kDa in the analysis of SDS-PAGE/silver staining (data not shown). The observed molecular mass of the deglycosylated soluble receptor was consistent with its calculated molecular weight from the primary structure (14), as well as the apparent molecular mass of the deglycosylated PLA_2-I receptor protein purified from bovine corpus luteum membranes (13). As shown in Fig. 1, [^{125}I]PLA_2-I binding

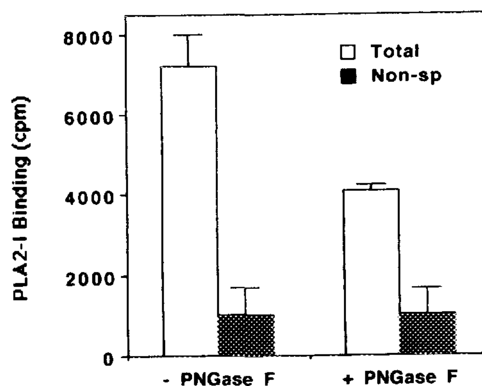

Fig. 1. Effect of PNGase F treatment of a recombinant soluble PLA_2-I receptor on the PLA_2-I binding activity.
Recombinant soluble PLA_2-I receptor (1 μg) was incubated with or without 1.2 U of PNGase F for 18 hr at 37°C. The receptor (10 ng) was then incubated with 1 nM [^{125}I]PLA_2-I for 2 hr at 4°C in the absence (total binding) or the presence (non-specific binding) of 500 nM unlabeled PLA_2-I. Data are expressed as mean ± S.E. performed in triplicate.

Table 1. Scatchard analysis of [^{125}I]PLA_2-I binding to the tunicamycin-treated BSMC

	Kd (nM)	Bmax (sites/10^6 cells)
None	0.38	1361
Tunicamycin	0.36	710

BSMC were incubated with or without 50 ng/ml of tunicamycin for 24 hr. After washing, the cells were incubated with increasing concentrations (0.2 - 6.0 nM) of [^{125}I]PLA_2-I for 2 hr at 4°C. The kinetic parameters were calculated from Scatchard plot that showed the presence of a single specific binding site for PLA_2-I.

activity was reduced about 45% after treatment with PNGase F, suggesting the important role of the N-linked oligosaccharides in the optimum binding of PLA_2-I.

The glycoprotein nature of the PLA_2-I receptor was further examined using intact cell systems. Cultured BSMC were found to possess a specific PLA_2-I receptor that did not recognize immature proPLA_2-I as well as PLA_2-II (data not shown). The effect of tunicamycin on the PLA_2-I binding was examined in BSMC, since this reagent is often used to block the N-glycosylation machinery under the synthesis of glycoproteins (15). Treatment of BSMC with tunicamycin for 24 hr resulted in a dose-dependent suppression of the PLA_2-I binding activity, with a half-maximum inhibition occurring at 20 ng/ml (data not shown). Tunicamycin (100 ng/ml) blocked about 70% of the ligand binding without affecting cellular DNA contents (data not shown). Scatchard analysis for the PLA_2-I binding in BSMC revealed that treatment with 50 ng/ml tunicamycin induced ca. 50% decrease in the number of PLA_2-I receptor without affecting its binding affinity for the ligand (Table 1), indicating that the removal of N-linked oligosaccharides from the receptor molecules impairs the receptor function. These results suggest a pivotal role of N-linked glycans for the ligand recognition. Alternatively, N-linked carbohydrates may be important for the proper translocation of the PLA_2-I receptor protein from endoplasmic reticulum to the cell surface membranes, as the receptor number of epidermal growth factor in A431 cells was reduced in the presence of tunicamycin because of the blockade of intracellular translocation of nonglycosylated form (20).

To evaluate the sugar specificity that involves the receptor functions, the effects of a variety of lectins were

examined. Lectins are known to recognize specific saccharides and bind to specific carbohydrate moieties which are often located on the cell surface receptor glycoproteins. Several lectins were incubated with BSMC at 50 μg/ml prior to the addition of $[^{125}I]PLA_2$-I. In the lectins studied, inhibitory effect was observed by the addition of Wheat germ agglutinin (WGA), a lectin which exhibits binding recognition for N-acetylglucosamine and N-acetylneuraminic acid (21). WGA suppressed the binding with a dose-dependent manner, and its inhibitory effect was considerably prevented by the addition of 100 mM N-acetyl-D-glucosamine (GlcNAc) (Fig. 2), but not by the other monosaccharides (L-fucose, D-mannose, N-acetyl-galactosamine or N-acetylneuraminic acid), suggesting that sugar recognition mediates the WGA-induced inhibition of PLA_2-I binding. Other lectins (Dolichos lectin specific for N-acetylgalactosamine, Peanut lectin specific for D-galactose and Concanavalin A specific for mannose or glucose) did not affect the PLA_2-I binding activity (data not shown). Taken together, these results suggest that WGA can inhibit specific PLA_2-I binding via steric interference or by inducing a conformation change in the ligand binding site which significantly lowers the equilibrium binding affinity of the receptor for PLA_2-I.

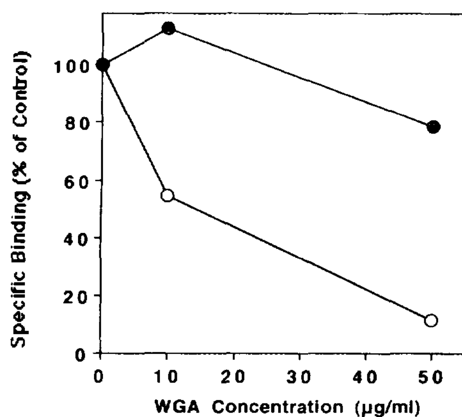

Fig. 2. Effect of WGA on the PLA_2-I binding to the receptor in BSMC. Confluent BSMC in a 6-well plate were incubated with WGA in the presence (●) or absence (○) of 100 mM GlcNAc for 15 min at 4°C. After the incubation, 3 nM $[^{125}I]PLA_2$-I was added, and the specific binding activity was measured. Data are expressed as mean values performed in duplicate.

In the present paper, we demonstrate the important roles of N-linked carbohydrates in the ligand recognition of the PLA_2-I receptor. The requirement of N-glycosylation for ligand binding was also observed in the thrombin receptor (22). Finally, increasing evidence suggests that oligosaccharides modify the expression and function of a variety of cell surface and soluble proteins (23). We are now conducting further studies to elucidate the precise structures of N-linked oligosaccharides in the PLA_2-I receptor that involves the optimum binding of PLA_2-I.

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