Phosphorylation of Neuroglycan C, a Brain-specific Transmembrane Chondroitin Sulfate Proteoglycan, and Its Localization in the Lipid Rafts*

Shinobu Yamauchi‡‡‡, Yoshihito Tokita‡, Sachiko Aono‡, Fumiko Matsui‡, Takuya Shuo‡‡, Hidenori Ito‡, Kanefusa Kato‡‡, Kohji Kasahara** and Atsuhiko Oohira‡‡‡

From the ©Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi 480-0392, the ©Department of Neurochemistry, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8550, the ©Nagoya City University Graduate School of Natural Sciences, Showa-ku, Nagoya 467-8501, and the **Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Bunkyo-ku, Tokyo 113-8613, Japan

Neuroglycan C (NGC) is a brain-specific transmembrane chondroitin sulfate proteoglycan. In the present study, we examined whether NGC could be phosphorylated in neural cells. On metabolic labeling of cultured cerebral cortical cells from the rat fetus with $^{32}$P, serine residues in NGC were radiolabeled. Some NGC became detectable in the raft fraction from the rat cerebrum, a signaling microdomain of the plasma membrane, with cerebral development. NGC from the non-raft fraction, not the raft fraction, could be phosphorylated by an in vitro kinase reaction. The phosphorylation of NGC was inhibited by adding to the reaction mixture a recombinant peptide representing the ectodomain of NGC, but not by adding a peptide representing its cytoplasmic domain. NGC could also be labeled by an in vitro kinase reaction using $[\gamma-32P]GTP$ as well as $[\gamma-32P]ATP$, and this kinase activity was partially inhibited by 5,6-dichloro-1-$\beta$-D-ribofuranosylbenzimidazole, a selective inhibitor of casein kinase II. In addition to the intracellular phosphorylation, NGC was also phosphorylated at the cell surface by an ectoprotein kinase. This is the first report to demonstrate that NGC can be phosphorylated both intracellularly and pericellularly, and our findings suggest that a kinase with a specificity similar to that of casein kinase II is responsible for the NGC ectodomain phosphorylation.

Proteoglycans are a group of proteins bearing sulfated glycosaminoglycans. They are located not only in the extracellular matrix as secretory molecules but also at the cell surface as transmembrane components or glycosylphosphatidylinositol-anchored molecules in various animal tissues including the central nervous system.

In the vertebrate central nervous system, there are many species of proteoglycan with different structural features. Each neural proteoglycan shows a particular spatiotemporal expression pattern in the brain, suggesting that it would function in particular phases of brain development. In fact, it has become clear that neural proteoglycans are involved in various cellular events including neural cell proliferation, cellular differentiation, neurite outgrowth, path finding, and synaptogenesis (1–9). In addition, it has been shown that the expression and metabolism of neural proteoglycans change after brain injury and are altered under some pathological conditions such as Alzheimer's disease (10–13).

Recently, we found a novel transmembrane chondroitin sulfate proteoglycan, named neuroglycan C (NGC), in the developing rat brain (14). To date, rat, mouse, and human NGC have already been cloned, and their expression is reported to be restricted to the central nervous system (14–16). The core protein consists of five structurally different domains: an N-terminal domain to which chondroitin sulfate is attached, an acidic amino acid cluster, a cysteine-rich domain with a single epidermal growth factor-like motif, a transmembrane domain, and a C-terminal cytoplasmic domain. Furthermore, there are some consensus sequences for phosphorylation by casein kinase II (CKII) in the ectodomain and by protein kinase C (PKC) in the cytoplasmic domain of the NGC core protein.

Protein phosphorylation and dephosphorylation are pivotal steps in signal transduction and play a key role in the regulation of many cellular processes (17). NGC would participate in signal transduction through its phosphorylation. Many signal-transducing molecules are concentrated in a particular microdomain of the plasma membrane. This microdomain is abundant in glycosphingolipids and cholesterol and is referred to as lipid rafts, caveolae membranes, detergent-resistant membranes, or detergent-insoluble glycosphingolipid-enriched domains (18–24). It has been suggested that the lipid rafts play an important role not only in the transport of membrane components but also in signal transduction (18, 19, 24). Therefore, it can be expected that NGC, as a signaling molecule, exists in the lipid rafts for effective signal transduction.

In this paper, we report that serine residues in the NGC core protein were phosphorylated in primary cultured rat neocortical cells and that some NGC became localized in the lipid rafts prepared from the rat cerebrum with cerebral development. In addition, we show that NGC can be phosphorylated by ectoprotein kinase activity at the surface of cerebral cortical cells in culture.

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The abbreviations used are: NGC, neuroglycan C; CHase ABC, protease-free chondroitinase ABC; CKI and CKII, casein kinase I and II, respectively; DBB, 5,6-dichloro-1-$\beta$-D-ribofuranosylbenzimidazole; GST, glutathione S-transferase; MES, 2-(N-morpholino)ethanesulfonic acid; PKC, protein kinase C; PVDF, polyvinylidene difluoride; RPTP, receptor-like protein tyrosine phosphatase.
**Experimental Procedures**

**Materials**—The following antibodies were used: a polyclonal anti-NGC antibody (15), a monoclonal anti-phosphacan/RPTP ζ/β antibody 6B4 (25), a polyclonal anti-N-syndecan antibody (26), and a monoclonal anti-flotillin antibody (Transduction Laboratories, Lexington, KY). The following protein kinase inhibitors were used: staurosporine (Wako Chemicals, Osaka, Japan), which is a serine/threonine kinase inhibitor with a broad specificity; 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; BIO MOL Research Labs, Inc., Plymouth Meeting, PA), which is a selective inhibitor of CKII with a half-maximal inhibitory concentration (IC₅₀) of 4–10 μM (27, 28); heparin (Sigma), which is an inhibitor of caspase kinase I (CKI; IC₅₀ = 24 μg/ml) and CKII (IC₅₀ = 0.15 μg/ml) (29, 30); GF190920X (BIO MOL Research Labs), which is a selective inhibitor of PKC with an IC₅₀ of 10 nM (31); KN-62 (Sigma), which is a selective inhibitor of PKC-δ; and calyculin A, a specific inhibitor of protein kinase C (36). The distribution of flotillin was investigated by immunoblotting using an anti-flotillin monoclonal antibody. Proteoglycans were also detected by immunoblotting using the anti-proteoglycan antibodies described above.

**Preparation of Recombinant NGC Core Protein Peptides**—To produce recombinant rat NGC polypeptides as glutathione S-transferase (GST) fusion proteins, cDNA fragments encoding the chondroitin sulfate attachment domain (amino acid 33–259) and cytoplasmic domain (448–544) of rat NGC were subcloned into pGEX 4T-1 (Amersham Biosciences) or plasmids were introduced into E. coli 4T-1 (Amersham Biosciences). The plasmids were introduced into E. coli using electroporation and the expression of fusion proteins was induced in the presence of 1 mM isoprpyl-β-D-thiogalactopyranoside. Cells were lysed in phosphate-buffered saline by mild sonication, and then Triton X-100 was added to the lysate to a final concentration of 1% with gentle mixing. After centrifugation of the mixture at 10,000 × g for 5 min at 4 °C, the supernatant was applied to a glutathione-Sepharose 4B (Amersham Biosciences) column, and proteins bound to the beads were eluted with elution buffer containing 1 mM glutathione. Affinity-purified fusion proteins, cDNA fragments encoding the chondroitin sulfate attachment domain (amino acid 33–259) and cytoplasmic domain (448–544) of rat NGC were subcloned into pGEX 4T-1 (Amersham Biosciences) or plasmids were introduced into E. coli using electroporation and the expression of fusion proteins was induced in the presence of 1 mM isoprpyl-β-D-thiogalactopyranoside. Cells were lysed in phosphate-buffered saline by mild sonication, and then Triton X-100 was added to the lysate to a final concentration of 1% with gentle mixing. After centrifugation of the mixture at 10,000 × g for 5 min at 4 °C, the supernatant was applied to a glutathione-Sepharose 4B (Amersham Biosciences) column, and proteins bound to the beads were eluted with elution buffer containing 1 mM glutathione. Affinity-purified fusion proteins were dialyzed against 30 mM HEPES, pH 7.5, at 4 °C for 20 h at 36,000 rpm at 4 °C. The supernatants were collected from the bottom of the gradient.

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**In Vitro Phosphorylation of Recombinant NGC Peptides**—CKII purified from rat liver (Promega Co., Madison, WI) and PKC purified from rat brain (Promega Co.) were used for in vitro phosphorylation of recombinant NGC peptides according to the instructions provided. Reconstituent GST fusion polypeptides representing the chondroitin sulfate attachment region and the cytoplasmic region of NGC were dissolved with the kinase reaction buffers in the presence of CKI or PKC. The phosphorylation reactions were started by the addition of [γ-³²P]ATP. After incubation for 10 min at room temperature, the reactions were stopped by the addition of the SDS sample buffer for SDS-PAGE, and the mixtures were boiled for 3 min. The samples were subjected to SDS-PAGE followed by autoradiography and/or immunoblotting with anti-NGC antibody. For immunoprecipitation of NGC from the bottom fractions of the sucrose density gradients, the sample was diluted with an equal volume of buffer E and processed in a way similar to that from the lipid raft fractions.

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was performed according to the method of Ito et al. (39). ³²P-Labeled NGC was subjected to SDS-PAGE and then transferred electrophoretically from the gel to a polyvinylidene difluoride (PVDF) membrane. The protein band containing phosphorylated NGC was cut out and hydrolyzed in 6 M hydrochloric acid at 110 °C for 1 h. The hydrolysate was evaporated and resuspended in 5 ml of a formate/acetate buffer (formic acid:acetic acid:0.1 M hydrochloric acid) at 110 °C for 1 h. The hydrolysate was evaporated and resuspended in 5 ml of a formate/acetate buffer (formic acid:acetic acid:0.1 M hydrochloric acid) at 110 °C for 1 h.

**Preparation of Lipid Rafts**—Lipid rafts were prepared by two different methods. The first method included the treatment of tissues with Triton X-100 (34). Rat cerebra at various ages were homogenized using a Teflon glass homogenizer in 9 volumes of 25 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl and 1 mM EGTA (buffer A). The lysate was brought to a concentration of 40% (w/v) sucrose by the addition of 80% sucrose. A linear sucrose gradient (5–30%) in buffer A without Triton X-100 was layered over the lysate. The gradient was centrifuged for 16–20 h at 36,000 rpm at 4 °C in a Hitachi RPS40T rotor. 10 fractions were collected from the bottom of the gradient.

The other method used to prepare lipid rafts involves the homogenization of tissues in an alkaline buffer (35). The cerebella of 22-day-old Sprague-Dawley rats were homogenized using a Teflon glass homoge-
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RESULTS

Phosphorylation of Serine Residues on NGC in Primary Cultures of Neocortical Cells—To test whether NGC is phosphorylated in primary cultures of fetal rat cerebral cortical cells, cells were incubated with $^{32}$P in the presence or absence of 1 mM Na$_2$HPO$_4$. NGC immunoprecipitated from the cell lysates was subjected to SDS-PAGE before and after treatment with CHase ABC followed by electrotransfer to a PVDF membrane. Radiolabeled materials were visualized by autoradiography of the membrane (Fig. 1A, left panel), and the NGC protein was detected by immunoblotting with polyclonal anti-NGC antibody (Fig. 1A, left panel). As reported previously (14), NGC was detected as a 150 kDa band before CHase digestion and as a 120 kDa band after CHase digestion. However, a significant amount of the 120 kDa band was visible even without the CHase ABC treatment. NGC is a part time proteoglycan, and the amount of a non-proteoglycan form without chondroitin sulfate chains increases gradually with the brain development (16). The radioactive bands coincided with the immunolabeled NGC bands, indicating that NGC is phosphorylated in the neocortical cells. Addition of unlabeled phosphate to a final concentration of 1 mM to the culture medium completely prevented the radiolabeling of NGC (Fig. 1A). Phosphoamino acid analysis of radiolabeled NGC by two-dimensional electrophoresis revealed that the radioactivity was detected exclusively at the position for phosphoserine (Fig. 1B).

Localization of NGC to the Lipid Rafts with the Maturation of the Brain—Many signaling molecules are concentrated in the lipid rafts, a signaling microdomain of the plasma membrane, where phosphorylation and dephosphorylation occur for signal transduction. Therefore, it can be expected that NGC, as a signaling molecule, exists in the lipid rafts. To examine the localization of NGC in the lipid rafts during the development of the brain, the rat cerebrum at defined developmental stages between postnatal day 4 (P4) and adulthood were analyzed by Triton X-100 extraction followed by sucrose density gradient ultracentrifugation. The localization of other neural transmembrane proteoglycans, phosphacan/RPTP$\beta$ antibody (B), and monoclonal anti-N-syndecan antibody (C), after digestion with CHase ABC. Flotillin was used as a marker protein of the brain lipid raft fraction. The positions of molecular mass markers are indicated at the right of the panels.
fraction in the brain (36), was detected in fractions 4–6 of the sucrose density gradient at all developmental stages examined. Most NGC was recovered in the bottom fraction, not in the lipid raft fraction, at early postnatal stages (P4, data not shown, and P12 in Fig. 2A). However, some was detectable in the lipid raft fraction around 3 weeks after birth (P22 and adult in Fig. 2A). Similarly, a large population of phosphacan/RPTP \(\beta\) was recovered in the lipid raft fraction after P22 (Fig. 2B), whereas \(N\)-syndecan was not detected in the lipid raft fraction at any developmental stage examined (Fig. 2C).

The presence of these neural proteoglycans in the lipid raft fraction prepared from the P22 rat cerebrum was then examined by preparing the lipid rafts using an alkaline solution instead of the detergent. The result was essentially the same as that obtained by the detergent-based method; both NGC and phosphacan/RPTP \(\beta\) were detected in the lipid raft fraction, but \(N\)-syndecan was not (data not shown). Taken together, these results indicate that some NGC as well as phosphacan/RPTP \(\beta\) is present in the lipid rafts of the mature brain and that \(N\)-syndecan is absent from the lipid rafts at all developmental stages.

NGC in the Lipid Rafts Is Not Phosphorylated by an in Vitro Kinase Reaction—To examine whether a protein kinase capable of phosphorylating NGC is associated with the proteoglycan, the immunoprecipitation of NGC was carried out both with the raft fraction and with the non-raft fraction, or the bottom fraction of the sucrose density gradient, of the P22 samples. An in vitro kinase reaction of the immunoprecipitates in the presence of \(\gamma^{32}\)P-ATP demonstrated that radioactive bands corresponding to the NGC bands were present only in the non-raft samples (Fig. 3A). Although NGC could be immunoprecipitated from the raft fraction, it was not phosphorylated by the in vitro kinase reaction (Fig. 3A, raft). Phosphoamino acid analysis of \(32\)P-labeled NGC of the non-raft fraction revealed radioactivity at the position of phosphoserine, not from the raft fraction, of the mature rat cerebrum can be phosphorylated by a protein kinase associated with NGC.

A Recombinant Ectodomain of NGC Inhibits the In Vitro Kinase Reaction of Endogenous NGC—To obtain a clue as to the phosphorylation site on NGC, we tried to inhibit the in vitro kinase reaction competitively by adding recombinant GST fusion polypeptides representing particular regions of the NGC core protein (Fig. 4A). In this series of experiments, NGC was immunoprecipitated from the lysate of the membrane fraction prepared from P22 rat cerebrum, and the immunoprecipitate was subjected to an in vitro kinase reaction with \(\gamma^{32}\)P-ATP in the presence of GST-NGCect, GST-NGCcp, or GST at the indicated concentrations followed by SDSPAGE after CHase ABC digestion. Radiolabeled NGC was detected by autoradiography (upper panels, \(32\)P). NGC protein was visualized by immunoblotting using anti-NGC antibody (lower panels, \(\alpha\)NGC). GST-NGCect added to the reaction mixture was intensely labeled with \(32\)P (left lower panel).

Unexpectedly, the addition of a polypeptide representing the chondroitin sulfate attachment region of NGC (GST-NGCect) inhibited the phosphorylation of endogenous NGC in a dose-dependent manner (Fig. 4B, left upper panel). The phosphorylation was inhibited completely at 40 \(\mu\)M GST-NGCect. Instead, GST-NGCcp added to the reaction mixture was intensely labeled with \(32\)P (Fig. 4B, left lower panel). Neither a recombinant polypeptide representing the cytoplasmic region of NGC (GST-NGCcp) nor GST inhibited the in vitro kinase reaction for endogenous NGC (Fig. 4B, center and right panels). These findings suggest that the chondroitin sulfate attachment region of the NGC ectodomain can be phosphorylated by a protein kinase coprecipitated with NGC from the detergent-solubilized membrane fraction of the cerebrum.

Phosphopeptide Mapping of NGC by CNBr Cleavage—To determine the phosphorylation sites on NGC, we performed CNBr cleavage of NGC prelabeled with \(32\)P by the in vitro kinase reaction. Four phosphopeptides, GST-NGCect, GST-NGCcp, with molecular sizes of \(\approx 100, 60, 38\), and \(24\) kDa were detected upon SDSPAGE followed by autoradiography (Fig. 5A). The smallest band was shown to have the N-terminal amino acid sequence of GRFPGPS (14), which represents the amino acid sequence of rat NGC beginning from Gly\(^{232}\). Considering the specificity of the chemical cleavage of peptides with CNBr (40), the 24-kDa peptide should represent the part of the NGC core protein from Gly\(^{232}\) to Met\(^{336}\) (Fig. 5B), which contains two potential phospho-
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Phosphorylation sites (Ser255 and Ser276) by CKII. The larger peptides may be the partial degradation products of NGC by CNBr cleavage at different methionine residues.

As described above, a protein kinase activity coprecipitated with endogenous NGC could phosphorylate the chondroitin sulfate attachment region (amino acids 33-259) of the NGC ectodomain (Fig. 4B). The peptide portion from Gly232 to Leu259 of the chondroitin sulfate attachment region overlaps with the 24-kDa phosphorylated CNBr peptide, suggesting that the phosphorylation sites of NGC exist on this peptide.

Characterization of the NGC Kinase Activity—The chondroitin sulfate attachment region of the NGC core protein contains some consensus sequences for phosphorylation by CKII. Therefore, we first tried to phosphorylate GST-NGCect using a commercially available CKII in the presence of \([\gamma-\text{32P}]\text{ATP}\). CKII efficiently phosphorylated GST-NGCect, but not GST-NGCcpe, which could be phosphorylated by a commercially available PKC (Fig. 6A) or GST (Fig. 6B). These results suggest that the chondroitin sulfate attachment region is phosphorylated by CKII or a kinase with a specificity similar to that of CKII in the brain.

To characterize further the NGC kinase activity, we examined the effects of several protein kinase inhibitors on the phosphorylation of NGC in the in vitro kinase reaction in the presence of \([\gamma-\text{32P}]\text{ATP}\). Staurorosporine, a serine/threonine protein kinase inhibitor with a relatively broad specificity, inhibited the NGC kinase activity at a concentration of lower than 1 \(\mu\text{M}\) (Fig. 6C). DBB, a CKII-specific inhibitor with an IC_{50} of 4-10 \(\mu\text{M}\), partially inhibited the phosphorylation of NGC at concentrations around IC_{50}. Heparin, an inhibitor of CKI (IC_{50}, 24 \(\mu\text{g/ml}\)) and CKII (IC_{50}, 0.15 \(\mu\text{g/ml}\)), also partially inhibited the phosphorylation of NGC, GF109203X, a specific inhibitor of PKC with an IC_{50} of 10 \(\mu\text{M}\), did not inhibit the NGC kinase activity at 10 \(\mu\text{M}\) but partially inhibited it at extremely high concentrations (Fig. 6C). Other reagents, KN-62, which is a specific inhibitor of Ca\(^{2+}\)/calmodulin-dependent kinase II, and CKI-7, which is a specific inhibitor of CKI, had no effect on the phosphorylation of NGC even at concentrations 10 times higher than their IC_{50} or K_i (data not shown).

All of the results described above suggest that NGC is phosphorylated by CKII or a kinase with a specificity similar to that of CKII. Because CKII is one of only a few kinases that can utilize GTP as well as ATP as a phosphate donor (30), we tried the phosphorylation of NGC in the in vitro kinase reaction in the presence of \([\gamma-\text{32P}]\text{GTP}\). As expected, NGC could be labeled with \(^{32}\text{P}\), although to a lesser extent than in the presence of \([\gamma-\text{32P}]\text{ATP}\). DRB again partially inhibited the phosphorylation at concentrations enough to inhibit completely the activity of the typical type of CKII, but heparin did not inhibit the activity (Fig. 6D). From these findings, it is postulated that the phosphorylation of NGC occurs in its chondroitin sulfate attachment region with a kinase that has a character similar, but not identical, to that of CKII.

Phosphorylation of NGC Takes Place Both Intracellularly and Extracellularly—Besides intracellular protein kinases, ectoprotein kinases acting at the surface of intact cells have been characterized (42-47). We examined whether the NGC ectodomain can be a substrate for ectoprotein kinases. Primary cultured cortical cells were incubated with \([\gamma-\text{32P}]\text{ATP}\) in the presence of 1 \(\mu\text{M}\) unlabeled phosphate. Because \([\gamma-\text{32P}]\text{ATP}\) does not penetrate the plasma membrane, phosphorylation of cell surface proteins by ectoprotein kinases can be detected selectively by this method. Both the 150 kDa intact NGC band and the 120 kDa NGC core glycoprotein band were radiolabeled under these conditions, indicating that NGC can be phosphorylated by an ectoprotein kinase (Fig. 7).

As shown in Fig. 1, NGC was labeled metabolically with \(^{32}\text{Pi}\) in primary cultures of fetal rat neocortical cells. However, this does not necessarily mean that NGC is phosphorylated intracellularly. Cells incubated with \(^{32}\text{P}\) produce \([\gamma-\text{32P}]\text{ATP}\), which can be released into the culture medium. Therefore, even when \(^{32}\text{P}\) is used, cell surface proteins can be radiolabeled by an ectoprotein kinase activity (45).

Then we examined whether NGC phosphorylation takes place both intracellularly and extracellularly. When cells were incubated with \([\gamma-\text{32P}]\text{ATP}\) in the presence of 1 \(\mu\text{M}\) unlabeled phosphate, NGC was radiolabeled (Fig. 8A, lane 2). This labeling was inhibited completely by adding 1 \(\mu\text{M}\) cold ATP to the culture (Fig. 8A, lane 3). When cells were incubated with \(^{32}\text{P}\), \(^{32}\text{P}\)-labeled NGC was still detectable even in the presence of 1 \(\mu\text{M}\) cold ATP (Fig. 8B, lane 2), the same concentration of cold ATP used for the complete inhibition of NGC radiolabeling by the ectoprotein kinase activity. These results clearly indicate that NGC can be phosphorylated intracellularly as well as at the cell surface.

DISCUSSION

In the present study, we obtained the first direct evidence that NGC, a brain-specific transmembrane proteoglycan, can be phosphorylated not only intracellularly but also at the cell surface in cultured cerebral cortical cells (Fig. 7). A protein kinase activity that phosphorylates serine residues in the NGC core protein was coimmunoprecipitated with NGC from the lysates of membrane fractions prepared from the brain and had properties similar to those reported for CKII. Although some NGC was recovered in the lipid rafts, a signaling microdomain of the plasma membrane, from mature brain, the NGC kinase activity was not detected in this microdomain at all, indicating that NGC in the lipid rafts occurs in a way different from that in other cellular membranes.

Two observations suggest that NGC phosphorylation occurs in the ectodomain, not the cytoplasmic domain. First, the phosphorylation of NGC in the in vitro kinase reaction was inhib-
ited by adding a recombinant peptide representing the chondroitin sulfate attachment region of the NGC ectodomain to the reaction mixture in a concentration-dependent manner but not by adding a recombinant peptide representing the cytoplasmic domain of NGC (Fig. 4). Moreover, the recombinant ectodomain of NGC added exogenously was phosphorylated efficiently in the in vitro kinase reaction (Fig. 4). Second, NGC can be phosphorylated by incubating cultured neural cells with $^{32}$P-ATP in the presence of 1 mM Na$_2$HPO$_4$ (Fig. 7), indicating that an ectoprotein kinase phosphorylates the ectodomain of NGC. It has been reported that some transmembrane proteins, such as T-lymphocyte surface proteins (48) and amyloid precursor protein (49), are phosphorylated on their ectodomains at two distinct cellular locations, in the cells and at the outer surface, just as in the case of NGC. The phosphorylation of the ectodomains of these proteins is considered to be a mechanism to modify molecular interactions at the cell surface. NGC has been shown to interact with tenascin through its acidic cluster (50) and with ErbB3 tyrosine kinase through the epidermal growth factor motif of the ectodomain. Although no molecules have been identified to interact directly with the chondroitin sulfate attachment region of the NGC ectodomain which is supposed to be phosphorylated, the ectodomain phosphorylation of NGC could change the cellular physiology of the NGC-expressing cells by adding a recombinant peptide representing the cytoplasmic domain of NGC (Fig. 4). Moreover, the recombinant ectodomain of NGC added exogenously was phosphorylated efficiently in the in vitro kinase reaction (Fig. 4). 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**Fig. 8.** Extracellular and intracellular phosphorylation of NGC in primary cultures of cerebral cortical cells. Cultured cerebral cortical cells were incubated with \([\gamma^{32}P]ATP\) for 15 min at 37 °C (A) or \(32P\) for 4 h at 37 °C (B) in the presence of 1 mM NaHPO\(_4\) and/or 1 mM cold ATP. NGC was immunoprecipitated from the cell lysates with anti-NGC antibody and separated by SDS-PAGE after treatment with CHase ABC. Proteins were electrotroduced to a PVDF membrane. Phosphorylated NGC was detected by autoradiography (lanes marked \(32P\)). NGC protein was visualized by immunostaining with anti-NGC antibody (lanes marked αNGC). The positions of molecular mass markers are indicated on the left of the panels.

Through modification of the cellular microenvironment, many signal-transducing molecules are concentrated in a particular microdomain of the plasma membrane referred to as lipid rafts. Therefore, the lipid rafts are thought to be implicated in signal transduction (19, 20, 24). We demonstrated that some NGC and phosphacan/RPTP \(ζ/β\) became localized in the lipid rafts in the mature brain (Fig. 2). This is the first report to show the existence of neural proteoglycans in the lipid raft fraction. RPTP \(ζ/β\) functions as a signal-transducing molecule for some heparin-binding growth factors such as pleiotrophin (HB-GAM) and midkine to mediate cell migration and neurite extension (58, 59). Phosphacan, an mRNA splicing product that represents the entire extracellular domain of RPTP \(ζ/β\), is a high affinity ligand of TAG-1, a glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule which exists mostly in the lipid rafts. In primary cultures of cerebellar granule cells, the addition of phosphacan inhibits neurite extension (unpublished observation) and induces the activation of Lyn, a src family kinase, through the cross-linking of TAG-1 (60). Thus, phosphacan/RPTP \(ζ/β\) are considered to regulate neuronal cell behavior in the lipid rafts as both a receptor and a ligand. The signaling cascade involving NGC is not known at present, but there is a possibility that NGC serves as a signal-transducing molecule in the lipid rafts. It is worth noting that not all of the neural transmembrane proteoglycans are found in the lipid rafts. N-Syndecan, another proteoglycan-type receptor for some heparin-binding growth factors (61), was not recovered in the lipid raft fraction from the cerebrum at any of the developmental stages examined (Fig. 2). It has been reported that growth factor binding to receptors not only induces the activation of signal transduction pathways but also translocates the receptor to the lipid rafts (62–64). Therefore, the present work does not rule out the possibility that N-syndecan becomes localized in the lipid rafts upon binding to a particular ligand.

It is interesting that, although NGC recovered from the non-raft fraction could be phosphorylated by the \(in vitro\) kinase reaction, NGC recovered from the lipid raft fraction could not (Fig. 3A). NGC would not be associated with the NGC kinase in the lipid rafts, or an inhibitor of the kinase may be coprecipitated with NGC from the lipid raft fraction. Additionally or alternatively, NGC in the lipid rafts would be structurally different from NGC in the non-raft fraction. NGC molecules fully phosphorylated in the intracellular membrane system or in the non-raft plasma membrane would be sorted into the lipid rafts. For some proteins detected in lipid rafts, phosphorylation (or dephosphorylation) is implicated in the sorting into or out of the lipid rafts (62, 65).

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