Regulation of Syndecan-4 Phosphorylation in Vivo*

(Received for publication, November 21, 1997, and in revised form, February 16, 1998)

Arie Horowitz and Michael Simons‡

From the Angiogenesis Research Center, Cardiovascular Division, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Recent studies suggest that some of the heparan sulfate-carrying proteoglycans may directly participate in signaling via their cytoplasmic tail. The present investigation addresses the potential involvement of syndecan-4, a widely expressed transmembrane proteoglycan, in this process. We found that the cytoplasmic tail of syndecan-4 is phosphorylated on a single serine residue (Ser\textsuperscript{183}) in growth-arrested NIH 3T3 fibroblasts, with a stoichiometry of 0.3 mol Pi/mol syndecan-4. Treatment of the cells with a protein kinase C (PKC)-activating phospholipid (KPKYKK) lead to a 2.5-fold increase in Ser\textsuperscript{183} phosphorylation. This increase was inhibited by a generic PKC inhibitor but not by an inhibitor specific to the calcium-dependent conventional PKCs, suggesting that the cytoplasmic tail of syndecan-4 is phosphorylated by a calcium-independent novel PKC isoform. Application of 10–30 ng/ml basic fibroblast growth factor (bFGF) produced a 2–3-fold reduction in the phosphorylation of syndecan-4. Because treatment with the phosphatase inhibitor calyculin prevented the bFGF-induced decrease in syndecan-4 phosphorylation, the effect of bFGF appears to be mediated by a protein serine/threonine phosphatase type 1 or 2A. We conclude that the cytoplasmic tail of syndecan-4 is subject to in vivo phosphorylation on Ser\textsuperscript{183}, which is regulated by the activities of a novel PKC isoform and a bFGF-dependent serine/threonine phosphatase.

Although growth factor signaling generally occurs through specific high affinity receptors, several growth factors interact with additional membrane-anchored co-receptors. In particular, bFGF\textsuperscript{1} requires binding to a specific sequence of sulfated polysaccharides in the extracellular heparan sulfate glycosaminoglycan (GAG) chain (1) to bind to its high affinity receptor (2) and to exert its effect on target cells (3, 4). The current picture of the role of heparan sulfate in the binding mechanism of bFGF consists of dimerization of the growth factor (2, 5, 6), as well as direct heparan sulfate binding to the high affinity receptor (7, 8). Together, these events lead to receptor multi-

* This work was supported by National Institutes of Health Grant HL-53793 (to M. S.), National Institutes of Health Training Grant HL-07374 (to A. H.) and American Heart Association Scientist Development Grant 9730282N (to A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Cardiovascular Div., RW453, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-5364; Fax: 617-975-5201; E-mail: msimons@bidmc.harvard.edu.

1 The abbreviations used are: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; PVDF, polyvinylidene fluoride.
whether the syndecan-4 molecule itself is subject to phosphorylation and whether this phosphorylation is affected by bFGF binding to the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calcinulin, chelerythrine, PMA, and bFGF were purchased from Sigma. G6 6976 was purchased from Calbiochem (La Jolla, CA). Chelerythrine, PMA, and G6 6976 were dissolved in MeSO3.

**Isolation of Syndecan-4 Core Proteins—**NIH 3T3 cells (American Type Culture Collection, Bethesda, MD) were grown to confluence in 100-mm petri dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Life Technologies Inc.) at 37 °C in a 5% CO2 humidified atmosphere. The cells were harvested by scraping in 1 ml of lysis buffer (150 mm NaCl, 20 mm NaF, 20 mm Na4P2O7, 5 mm EDTA, 5 mm EGTA, 1 mm Na3VO4, 1 mm phenylmethylsulfonyl fluoride, 1% Triton X-100, 50 mm HEPES, pH 7.4). The lysate was cleared by centrifugation at 9000 g for 30 min and then subjected to DEAE-chromatography as described (26). The eluates were dialyzed twice against 10 mm NH4HCO3, 1 mm EDTA, and resolved by SDS-PAGE on a 10% slab gel, and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was used as standard. The medium, trypsinization supernatant, and the extract fractions were precleared by adding 30 l of nonimmune rabbit serum (Life Technologies Inc.). After blocking in PBS containing 5% nonfat milk powder for 1 h at 22 °C, the membrane was incubated in the same solution supplemented with 1:3000 (v/v) dilution of either ectoplasmic or cytoplasmic syndecan-4 domain-specific antiserum for 2 h, washed with PBS, and incubated for 1 h in 5% milk powder-PBS containing 1:2000 diluted goat anti-rabbit IgG conjugated to peroxidase (Vector Laboratories, Burlingame, CA). The secondary antibody was detected, after an additional PBS wash, by chemiluminescence (Western Blot Chemiluminescence Reagent Plus, New England Nuclear). Molecular weights were estimated by comparison with the electrophoretic mobility of standards (Kaleidoscope Prestained Standards, Bio-Rad). Densitometry of digitized images of immunoblotted membranes (ScanJet 4c, Hewlett Packard) was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Phosphorylation of Syndecan-4 Cytoplasmic Tail**

To determine the presence and extent of phosphorylation of the syndecan-4 cytoplasmic tail, full-length heparan and chondroitin sulfate-carrying core proteins were isolated from serum-starved, [32P]-labeled NIH 3T3 cells. Autoradiography of NIH 3T3 GAG-lysed core proteins is shown in Fig. 1A (lane 1). To identify the syndecan-4 band, the autoradiographed membrane was probed with an antisem specific to the cytoplasmic tail of the syndecan-4 core protein (26). The immunoblotting highlighted a single band that ran at an approximate molecular mass of 36 kDa (Fig. 1A, lane 1). A similar syndecan-4 cytoplasmic tail-specific band was observed in the phosphorimager (Fig. 1A, syn-4) shown in A. Phosphoamino acids were identified by comparison with the electrophoretic mobility of nonradioabeled standards: P-Ser, Ser(P); P-Thr, Thr(P); P-Tyr, Tyr(P).
Phosphorylation of Syndecan-4 Cytoplasmic Tail

Electrophoretic mobility of phosphorylated serine (Fig. 1B). The syndecan-4 core protein sequence contains multiple serines (16 in the human syndecan-4 (27) and 15 in the rat (14)), all but one of which are located in the ectoplasmic domain. To determine which domain contains the phosphorylated serine, we exploited the susceptibility of the ectoplasmic domain of the syndecans to trypsinization (28). Thus the core protein of syndecan-4 was cleaved at the cell surface concurrently with the trypsin dissociation of the \(^{32}\)P-labeled, adherent cells from the culture plates.

By analogy with syndecan-1 (28), the trypsinization site is most likely between Arg\(^{147}\) and Thr\(^{148}\) preceding the transmembrane domain. Following trypsinization and detergent extraction, the cleaved syndecan-4 fragment was isolated by immunoprecipitation with the cytoplasmic tail-specific antiserum, which recognizes a 14-residue cytoplasmic sequence (26). As with the full-length core protein, the \(^{32}\)P-labeled immunoprecipitate was separated by SDS-PAGE and transferred to a membrane. The band routinely detected in the autoradiographs of these membranes migrated at an approximate molecular mass of 5 kDa, slightly less than the predicted 7-kDa size of the fragment encompassing the trypsinized transmembrane and cytoplasmic tails (Fig. 2A). This lower apparent molecular mass may have resulted from partial degradation during the isolation process or may reflect a higher electrophoretic mobility than the molecular mass standard used for estimating the band size. To verify the identity of this band, we reprobed the same membrane with the antisera that recognizes a 14-residue cytoplasmic sequence (26). Moreover, the band was quantified by densitometry. In cells treated by 10 and 30 ng/ml bFGF, the level of syndecan-4 expression was 85% (mean \(\pm\) S.D., \(n = 5\)) of that of the basal level. If, contrary to our assumption, the incorporation efficiency of \(^{35}\)S is higher than that of \(^{32}\)P, the bFGF-induced decrease in syndecan-4 phosphorylation could solely result from bFGF up-regulation of syndecan-4 synthesis. To address this possibility, the syndecan-4 expression levels in control and bFGF-treated cells were compared by immunoblotting cell lysates containing equal amounts of total protein. The syndecan-4 bands, which similar to immunoprecipitated samples (Fig. 2A) ran at an approximate molecular mass of 5 kDa, were detected with the antisera specific to the ectoplasmic domain, and the amount of protein in each band was quantified by densitometry. In cells treated by 10 and by 30 ng/ml bFGF, the level of syndecan-4 expression was 85% (Fig. 3B, inset) and 93% of the control cells, respectively.

The possible involvement of PKC in syndecan-4 phosphorylation is suggested by several reports on functional relationships between the kinase and the proteoglycan (18–20). To up-regulate PKC, cells were treated with the PKC-activating phorbol ester PMA (0.5 \(\mu\)M) during the last 5 h of the serum-free starvation. This treatment increased only the Ser\(^{183}\) phosphorylation of syndecan-4, without having a detectable effect.
on the phosphorylation of threonine or tyrosine residues in the cytoplasmic tail (Fig. 4A). The stoichiometry of the phosphorylation of syndecan-4 in the PMA-treated cells was 0.81 ± 0.33 (n = 3), close to 3-fold higher than the basal level. This result indicates that syndecan-4 is either a direct or an indirect PKC substrate.

To further examine the role of PKC in syndecan-4 phosphorylation, we applied the PKC-specific inhibitor chelerythrine to PMA-stimulated cells. The phosphorylation of syndecan-4 started to decline at chelerythrine concentrations above 1.5 μM and was reduced to an undetectable level at 6 μM chelerythrine (Fig. 4, B and C). The latter concentration is less than 10% of the IC_{50} of chelerythrine for the inhibition of protein tyrosine kinases (29). Although supporting the role of PKC in the phosphorylation of syndecan-4, these results do not identify the specific isozyme involved, because both PMA and chelerythrine affect all the four known calcium-dependent cPKCs, as well as the five calcium-independent nPKCs (30). To further narrow down the group of possible PKC isozymes, we applied the indolocarbazole Gö 6976, which selectively inhibits calcium-dependent PKC isozymes (31) to PMA (0.5 μM)-treated cells. The phosphorylation of syndecan-4 was not reduced, however, by Gö 6976 concentrations up to 100 nM, more than 10-fold its IC_{50} for cPKC (data not shown). It is likely, therefore, that the syndecan-4 cytoplasmic tail is phosphorylated by one of the nPKC isozymes.

DISCUSSION

We have shown that the cytoplasmic tail of syndecan-4 is phosphorylated in cultured fibroblasts and that the extent of its phosphorylation is determined by activities of a nPKC enzyme and a bFGF-activated phosphatase. The phosphorylation site was localized to Ser183, immediately upstream of a nine-amino acid segment involved in binding to and activation of PKC α (19). Phosphorylation of a cytoplasmic serine residue was previously detected in the cytoplasmic tail of syndecan-2 (23) and to a lesser extent in syndecan-1 (24). In our studies we observed a relatively high degree of syndecan-4 phosphorylation in growth-arrested cells, which could be further increased by treatment with PMA or decreased by bFGF. Because Ser^{183} is part of an invariant seven-residue sequence (KKDEGSY), these findings may be relevant to all four members of the syndecan family.

The PMA-induced increase in the phosphorylation of syndecan-4 and its decrease by chelerythrine strongly suggest the
involvement of PKC in this phosphorylation. Syndecan-4 could not be phosphorylated in vitro, however, by cPKC isozymes (21, 22). In agreement with this observation, we were unable to suppress the PMA-induced phosphorylation of syndecan-4 by a cPKC-specific inhibitor, pointing to the participation of a nPKC isozyme in the phosphorylation. Although the amino acid sequence around Ser183 in the cytoplasmic tail of syndecan-4 does not ideally fit a particular PKC isozyme-specific substrate sequence motif (32), it does contain the glycosyl residue that immediately precedes the phosphorylatable serine/threonine in the motifs of all the PKC isozymes. The nPKC isozymes δ, ε, and η were observed to be membrane-associated in NIH 3T3 fibroblasts (33), the same cell type used in the present study. The substrate sequence motif of PKCδ (AKRRKGFFYYGG, (32)) has the highest similarity among all PKC isozymes to the amino acid sequence around Ser183 in syndecan-4.

A phosphatase inhibitor reversed the bFGF-induced reduction in syndecan-4 phosphorylation observed in our study. This suggests that bFGF binding up-regulates a phosphatase and/or down-regulates a kinase involved in controlling the level of Ser183 phosphorylation. This effect could be mediated either through the bFGF high affinity tyrosine kinase receptor or through the syndecan-4 molecule. Tyrosine phosphorylation was reported to inhibit PKCδ (34), although an opposite stimulatory effect of this phosphorylation has also been observed (35). Alternatively, dimerization of bFGF molecules bound to heparan sulfate chains of adjacent syndecan-4 core proteins could cross-link these molecules. This would facilitate trans-phosphorylation of their cytoplasmic tails by a putative tail-associated phosphatase, similar to the association of tyrosine phosphatase to the cytoplasmic tails of growth factor receptors (10). Although the identity of the phosphatase cannot be determined from our data, its susceptibility to calyculin indicates that it is likely to be a serine/threonine phosphatase type 1 or 2A.

Although these findings demonstrate multi-factorial regulation of syndecan-4 cytoplasmic tail phosphorylation, the functional impact of this event is not known. Recent findings have suggested that syndecan-4 may play an important role in regulating the distribution and activity of PKCα. These functions are mediated via the oligomerization of a unique nine-amino acid domain (Leu186-Lys184) starting three residues downstream of the phosphorylated serine (19). The state of Ser183 phosphorylation may conceivably affect syndecan-4 oligomerization. Indeed, phosphorylation of a cysteine residue appended to the amino terminus of a Leu186-Lys184 synthetic peptide reduced the tendency of this peptide to oligomerize in vitro (20). The location of the phosphorylated cysteine is only two residues downstream of Ser183. Thus, the bFGF-induced phosphorylation of the syndecan-4 cytoplasmic tail on Ser183, which we have detected in vitro, may be required for oligomerization of the core protein, as well as for other possible processes. These may include co-assembly of syndecan and high affinity bFGF receptors into signaling complexes (9, 12), association with the actin cytoskeleton (17), and recruitment into focal adhesions (18). These and other potential consequences of syndecan-4 phosphorylation are currently being investigated.

Acknowledgment—We thank Dr. Colleen Sweeney Crovello for expert help with TLC.

REFERENCES

1. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) J. Biol. Chem. 267, 10337–10341
2. Yayon, A., Klagesbrun, M., Esco, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 841–848
3. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) Science 252, 1705–1708
4. Olwin, B. B., and Rapraeger, A. (1992) J. Cell Biol. 118, 631–639
5. Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C. M., and Waksman, G. (1995) Science 268, 432–436
6. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Science 271, 1116–1120
7. Kan, M., Wang, F., Xu, J., Crab, J. W., Hou, J., and McKeone, W. L. (1995) Science 259, 1918–1923
8. Brickman, Y. G., Ford, M. D., Small, D. H., Bartlett, P. F., and Nusrem, V. (1995) J. Biol. Chem. 270, 24941–24948
9. Krufka, A., Guimond, S., and Rapraeger, A. C. (1996) Biochemistry 35, 11111–11114
10. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Annu. Rev. Cell Biol. 10, 251–337
11. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L., and Loes, E. J. (1992) Annu. Rev. Cell Biol. 8, 365–393
12. Rapraeger, A. C. (1993) Curr. Opin. Cell Biol. 5, 844–853
13. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. Z., and Zhang, L. (1997) J. Clin. Invest. 99, 2062–2070
14. Kojima, T., Shworak, N. W., and Rosenberg, R. D. (1992) J. Biol. Chem. 267, 4870–4877
15. Carey, D. J., Stahl, R. C., Cizmeci-Smith, G., and Asundi, V. K. (1994) J. Cell Biol. 124, 161–170
16. Carey, D. J., Stahl, R. C., Tucker, B., Bendt, K. A., and Cizmeci-Smith, G. (1994) Exp. Cell Res. 214, 12–21
17. Carey, D. J., Bendt, K. M., and Stahl, R. C. (1996) J. Biol. Chem. 271, 15253–15260
18. Bacic, P. C., and Goetinck, P. F. (1995) Mol. Cell Biol. 6, 1503–1513
19. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 8133–8136
20. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 11805–11811
21. Prystafo, T., Ek, B., Ekman, P., Owens, R., Hook, M., and Johansson, S. (1995) Biochem. Mol. Biol. Int. 36, 793–802
22. Oh, E. S., Couchman, J. R., and Woods, A. (1997) Arch. Biochem. Biophys. 344, 67–74
23. Itano, N., Oguri, K., Nagayasu, Y., Kassano, Y., Nakanishi, H., David, G., and Tkvayama, M. (1996) Biochem. J. 315, 925–930
24. Reiland, J., Ott, V. L., Lebakken, C. S., Yeaman, C., McCarthy, J., and Rapraeger, A. C. (1996) Biochem. J. 319, 39–47
25. Gould, S. E., Upholt, W. B., and Kosher, R. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3271–3275
26. Shworak, N. W., Shirakawa, M., Mulligan, R. C., and Rosenberg, R. D. (1994) J. Biol. Chem. 269, 21204–21214
27. Kojima, T., Inazawa, J., Takamatsu, J., Rosenberg, R. D., and Saito, H. (1993) Biochem. Biophys. Res. Commun. 190, 814–822
28. Saunders, S., Jalkanen, M., and Bernfield, M. (1989) J. Cell Biol. 108, 1547–1556
29. Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990) Biochem. Biophys. Res. Commun. 172, 993–999
30. Nishizuka, Y. (1995) FASEB J. 9, 484–496
31. Martiny-Baron, G., Kastaniotis, M. G., Mischak, H., Blumberg, P. M., Kocha, G., Hug, H., Marne, D., and Schachtete, C. (1993) J. Biol. Chem. 268, 9194–9197
32. Nishikawa, K., Toker, A., Johnnes, F. J., Songyang, Z., and Cantley, L. C. (1997) J. Biol. Chem. 272, 952–960
33. Goodnight, J. A., Mischak, H., Kelch, W., and Mushinski, J. F. (1995) J. Biol. Chem. 270, 9991–10003
34. Denning, M. F., Dlugosz, A. A., Threadgill, D. W., Magnuson, T., and Yospa, S. H. (1996) J. Biol. Chem. 271, 5325–5331
35. Li, W., Mischak, H., Yu, J. C., Wang, L. M., Mushinski, J. F., Heidarman, M. A., and Pierce, J. H. (1994) J. Biol. Chem. 269, 2349–2352