Induction of fibronectin gene expression by inhibitors of protein phosphatase type 2B in normal and transformed fibroblasts

Jung-Hwa Rhew¹, Young-Ah Shin¹, Byung-Heon Lee², Rang-Woon Park¹ and In-San Kim^{1,3}

¹ Department of Biochemistry, School of Medicine, Kyungpook National University, 101 Dongin-Dong, Taegu, 700-422, Korea

² Department of Biochemistry, School of Medicine, Dongguk University, 707 Sukjang-dong, Kyungju, 780-714, Korea

³ Corresponding author: Tel, +82-53-420-6934; Fax, +82-53-422-1466; E-mail, iskim@bh.kyungpook.ac.kr

Accepted 15 April 1999

Abbreviations: FN, fibronectin; PKC, protein kinase C; PP2B, protein phosphatase type 2B; MEM, minimal essential medium; BSA, bovine serum albumin; FBS, fetal bovine serum; PMA, phorbol myristate acetate; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; TGF- β , transforming growth factor- β

Abstract

Two intracellular signal pathways mediated by cAMP and protein kinase C (PKC) were involved in the regulation of FN gene expression (Lee *et al.*, *Exp. Mol. Med.* 30: 240, 1998). In this study, a possible involvement of protein phosphatase-dependent pathways in the regulation of FN gene expression was investigated by using protein phosphatase type 2B (PP2B) inhibitors, cyclosporin A and ascomycin. Both cyclosporin A and ascomycin increased the levels of FN mRNA in WI-38 human lung fibroblasts and the SV40-transformed WI-38 cells but not in MC3T3-E1 osteoblasts. The expression of FN appears to increase from six hours up to 48 hours after treatment suggesting that it is not an immediate effect. In addition, this effect required a new protein synthesis. Neither cyclosporin A nor ascomycin affects the phorbol myristate acetate (PMA)-induced stimulation of FN gene expression and the same result occurred in vice versa suggesting the mechanism of PMA and cyclosporin A/ascomycin in the regulation of FN gene expression may share a common downstream pathway. Taken together, this study suggests that PP2B is involved in the regulation of FN gene expression in normal and transformed fibroblasts but not in osteoblasts.

Keywords: ascomycin, cyclosporin A, fibroblasts, fibronectin, protein phosphatase 2B

Introduction

Fibronectin (FN) is a large, extracellular matrix (ECM) glycoprotein that functions as both plasma and ECM protein. It is composed of two subunits of about 230 and 250 kDa that are cross-linked by disulfide bonds. FN serves as an attachment factor for cell-matrix and cell-cell interactions in various biological processes such as adhesion, differentiation, and migration (Rouslahti, 1981, 1988; Hynes, 1990). Many different FN polypeptides are generated from a single gene by alternative splicing (Borsi *et al.*, 1987; Norton and Hynes, 1987; Schwarzbauer *et al.*, 1987; Zardi *et al.*, 1987). FN has several domains known to bind a variety of matrix proteins such as type I collagen and also it has a specific amino acid sequence RGD that is recognized by a transmembrane receptor, integrin expressed on many cells (Hynes, 1987; Rouslahti and Pierschbacher, 1987). A variety of growth factors and cytokines are being evaluated as modulators of FN gene expression in many different cell types (Nernath *et al.*, 1980; Senger *et al.*, 1983; Allen-Hoffmann and Mosher, 1987; Almendral *et al.*, 1988; Blatti *et al.*, 1988; Bowlus *et al.*, 1991; Dean *et al.*, 1988, Dean *et al.*, 1989). As we previously reported (Lee *et al.*, 1996; Lee *et al.*, 1997; Lee *et al.*, 1998), two intracellular signal pathways mediated by cAMP and protein kinase C (PKC) are involved in the regulation of FN gene expression. These findings led us to investigate whether protein phosphatase-dependent pathways may also be responsible for FN gene expression. Here, we reported that ascomycin and cyclosporin A, inhibitors of serine/threonine protein phosphatase type 2B (PP2B), stimulated FN gene expression in WI-38 human lung fibroblasts and its SV-40 transformed derivative, WI-38VA13, but not in MC3T3-E1 osteoblasts.

Materials and Methods

Materials

Minimal essential medium (MEM), α -MEM, and fetal bovine serum (FBS) were purchased from Gibco/BRL (Gaithersburg, MD, U.S.A.). Bovine serum albumin (BSA), nonessential amino acids, pyruvic acid, phorbol-12-myristate-13-acetate (PMA), goat anti-human FN antibody, and cycloheximide (CHX) were from Sigma (St. Louis, MO, U.S.A.). Cyclosporin A and ascomycin were from BIOMOL (Plymouth Meeting, PA, U.S.A.). Protein A-Sepharose was from Pharmacia (Milwaukee, WI, U.S.A.).

Culture plastic wares were from Costar (Cambridge, MA, U.S.A.). Nitrocellulose membrane, [35 S]methionine, [32 P]dCTP (3,000 Ci/mmol) and random primed DNA labeling kit were from Amersham (Arlington, IL, U.S.A.). The ExpressHybTM hybridization solution was from Clontech (Palo alto, CA, U.S.A.).

Cell cultures

A normal human lung fibroblast cell line WI-38, an SV-40 virus-transformed derivative of WI-38, WI-38VA13, and a mouse calvarial osteoblastic cell line MC3T3-E1 were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). WI-38 and WI-38VA13 were cultured in MEM supplemented with 10% FBS. MC3T3-E1 was cultured in α -MEM supplemented with 10% FBS. Each medium used in this study was supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 0.1% nonessential amino acids (only for WI-38VA13), and 1 mM pyruvic acid (only for WI-38VA13). Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Radioactive labeling and immunoprecipitation

of FN

Confluent cells in 35-mm culture dishes were incubated with serum free media supplemented with 1% BSA and the indicated agents and then labeled for two hours with methionine-free MEM containing 30 μ Ci/ml of [35 S]methionine. Radiolabeled FN was immunoprecipitated as described previously (Lee et. al., 1996). Excess (3 mg) of goat anti-human FN antibody was added to the combined samples of medium and cell layer extracts containing the same amounts of radioactive protein and then incubated at 4°C for 1–2 h. The immune complexes were recovered by addition of 30 ml of protein A-Sepharose beads, and incubated with shaking for an additional 1 hour. The adsorbed proteins on Sepharose beads were dissolved in a 30 ml of electrophoresis sample buffer heated at 100°C for 4 min. Samples were analyzed by SDS-PAGE using a 6% gel, and subjected to fluorography.

Northern blot hybridizations

Total cellular RNA was isolated with acid guanidinium thiocyanate-phenol-chloroform as described previously (Chomczynski and Sacchi, 1987). Ten μ g of total cellular RNA per sample were fractionated by electrophoresis on a 0.8% agarose gel containing 2.2 M formaldehyde, trans-

Figure 1. Effect of ascomycin on fibronectin (FN) mRNA levels in WI-38. (A), WI-38VA13 (B) and MC3T3-E1 (C). Confluent cells were treated with the indicated concentrations of ascomycin for 24 h. Total RNA extraction and Northern blot analysis were performed as described in Materials and Methods. 18S, 18S ribosomal RNA.

Figure 2. Effect of cyclosporin A on fibronectin (FN) mRNA levels in WI-38. (A), WI-38VA13 (B), and MC3T3-E1 (C). Confluent cells were treated with the indicated concentrations of cyclosporin A for 24 h. 18S, 18S ribosomal RNA.

Figure 3. Time kinetics of fibronectin (FN) mRNA levels by cyclosporin A (A) and ascomycin (B) in WI-38. Cells were treated with cyclosporin A (1,000 ng/ml) or ascomycin (1,000 ng/ml) for the indicated periods of time. 18S, 18S ribosomal RNA; C, control.

Figure 4. Effect of cycloheximide on the cyclosporin A-induced fibronectin (FN) mRNA levels. Confluent WI-38 cells were pre-treated with cycloheximide (10 mg/ml) before treatment of cyclosporin A (1,000 ng/ml) for 24 h. 18S, 18S ribosomal RNA.

Figure 5. Effect of phorbol myristate acetate (PMA) alone or in combination with ascomycin or cyclosporin A on fibronectin (FN) mRNA levels in WI-38. Cells were treated with PMA (10 ng/ml) in combination with cyclosporin A (1,000 ng/ml) or ascomycin (1,000 ng/ml) for 24 h. 18S, 18S ribosomal RNA.

ferred to a nylon membrane followed by hybridization and subsequent washing as described by Sambrook *et al.* (1989). A cDNA probe was labeled with [32 P]dCTP using a random primed DNA labeling kit. The membrane was exposed to a X-ray film at -70°C . The cDNA probes used for hybridization were FH1, human FN cDNA (Kornblihtt, *et al.*, 1983) and 18S ribosomal RNA cDNA.

Results and Discussion

In order to determine the effect of a PP2B inhibitor ascomycin on FN mRNA levels in normal and transformed lung fibroblasts, WI-38 and WI-38VA13, respectively and in MC3T3-E1 osteoblasts, cells were exposed to ascomycin ranging from 1 to 1,000 ng/ml for 24 h and the steady state levels of FN mRNA were determined by Northern blot hybridization. Ascomycin increased FN mRNA levels in WI-38 and WI-38VA13 cells in a dose-dependent manner but not in MC3T3-E1 cells (Figure 1). Cyclosporin A, another inhibitor of PP2B, also increased

FN mRNA levels in WI-38 and WI-38VA13 cells but not in MC3T3-E1 cells (Figure 2). A cDNA for 18S ribosomal RNA was used to correct the amount of RNA loaded. We also tested okadaic acid and calyculin A, protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A) inhibitors, resulting in marked stimulation of FN gene expression in MC3T3-E1 cells but not in WI-38 and WI-38VA13 cells (data not shown). These results suggest that the regulation of FN gene expression by protein dephosphorylation is different depending on cell types.

To study the time kinetics of mRNA levels, confluent WI-38 cells were treated with 1,000 ng/ml of cyclosporin A or 1,000 ng/ml of ascomycin for the indicated periods of time. Post-treatment levels of FN mRNA began to increase from 6 h up to 48 h with both agents. (Figure 3). A protein synthesis inhibitor CHX was added 30 min before the addition of cyclosporin A. While CHX itself increased levels of FN mRNA in WI-38 cells, it blocked the cyclosporin A-induced increase of FN mRNA levels

(Figure 4), suggesting that a new protein synthesis is required for the induction of FN gene expression by cyclosporin A.

We have reported that PMA increases FN synthesis and gene expression in WI-38 and WI-38VA13 cells through the PKC-dependent pathway (Lee *et al.*, 1996). We have also found that PMA induces FN gene expression in MC3T3-E1 (data not shown). To investigate whether ascomycin and cyclosporin A effect the PMA-induced FN synthesis or vice versa, WI-38 cells were treated with 10 ng/ml of PMA alone or in combination with ascomycin or cyclosporin A. A lower concentration (1 ng/ml) of ascomycin and cyclosporin A which merely stimulated FN gene expression was used. FN gene expression was stimulated by PMA alone but not by either ascomycin or cyclosporin A alone at a given concentration (Figure 5). In addition, the PMA-induced stimulation was not enhanced by the combined treatment of PMA with either ascomycin or cyclosporin A (Figure 5). These results suggest that the mechanism of PMA and ascomycin/cyclosporin A in the regulation of FN gene expression may share a common downstream pathway.

This is the first report to show that PP2B is involved in the regulation of FN gene expression. Cyclosporin A and ascomycin are well-known immunosuppressants and their inhibitory action on calcineurin, a Ca^{2+} /calmodulin-dependent PP2B, is mediated by making complex to their intracellular receptors, cyclophilin and FK506 binding protein, respectively (Fruman *et al.*, 1994). Cyclosporin A has been widely used for the prevention of a graft rejection (Liu *et al.*, 1991). However, one of the major concerns is its potential nephrotoxicity causing interstitial fibrosis (Myers, 1986). Therefore, its effects on the transcription and synthesis of extracellular matrix proteins such as type I and IV collagen have been studied. Wolf *et al.* (1990) reported that cyclosporin A stimulated collagen synthesis and gene expression in kidney fibroblasts and tubular cells. Frizell *et al.* (1994) reported that another PP2B inhibitor FK506 also increased the expression of extracellular matrix genes. Here we reported that FN, one of the important extracellular matrix proteins, are also up-regulated by cyclosporin A and ascomycin suggesting that the increased synthesis of FN by them may contribute to the tissue fibrosis which is one of major side effects of these immunosuppressant drugs. Although the precise mechanism of the action of cyclosporin A and ascomycin on FN gene expression are uncertain, the results of this study provide an insight for understanding the regulatory mechanisms of FN gene expression in response to various extracellular stimuli. Indeed, a recent study provided evidences that cyclosporin A induced transforming growth factor- β (TGF- β) production which in turn enhanced tumor growth and the number of metastasis (Hojo *et al.*, 1999). Our results that cyclosporin A requires a new protein synthesis to induce FN synthesis suggest that the effect of cyclosporin A may act indirectly through TGF- β production.

In addition, our results showed that the regulation of FN expression by protein phosphatase seems to be cell-type specific since PP2B inhibitors such as cyclosporin A and ascomycin increased FN expression in fibroblasts but not in osteoblasts.

Acknowledgement

This work was supported by a grant of Kyungpook University Hospital and a grant BSRI 98-4403 from the Korean Research Foundation of the Ministry of Education provided in the program year of 1998.

References

- Allen-Hoffmann, B. L. and Mosher, D. F. (1987) Matrix assembly sites for exogenous fibronectin are decreased on human fibroblasts after treatment with agents which increase intracellular cAMP. *J. Biol. Chem.* 262: 14361-14365
- Almendral, J. M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol. Cell Biol.* 8: 2140-2148
- Bernath, V. A., Muro, A. F., Vitullo, A. D., Bley, M. A., Baranao, J. L. and Kornblitt, A. R. (1990) Cyclic AMP inhibits fibronectin gene expression in a newly developed granulosa cell line by a mechanism that suppresses cAMP-responsive element-dependent transcriptional activation. *J. Biol. Chem.* 264: 9069-9076
- Blatti, S. P., Foster, D. N., Ranganathan, G., Moses, H. L. and Getz, M. J. (1988) Induction of fibronectin gene transcription and mRNA is a primary response to growth-factor stimulation of AKR-2B cells. *Proc. Natl. Acad. Sci. USA* 85: 1119-1123
- Borsi, L., Carnemolla, B., Castellani, P., Rosellini, C., Vecchio, D., Allemanni, G., Chang, S. E., Taylor-Paradimitriou, J., Pande, H. and Zaldi, L. (1987) Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. *J. Cell. Biol.* 104: 595-600
- Bowlus, C. L., McQuillan, J. J. and Dean, D. C. (1991) Characterization of three different elements in the 5'-flanking region of the fibronectin gene which mediate a transcriptional response to cAMP. *J. Biol. Chem.* 266: 1122-1127
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159
- Dean, D. C., Blakeley, M. S., Newby, R. F., Ghazal, P., Hennighausen, L. and Bourgeois, S. (1989) Forskolin inducibility and tissue-specific expression of the fibronectin promoter. *Mol. Cell Biol.* 9: 1498-1506
- Dean, D. C., Newby, R. F. and Bourgeois, S. (1988) Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor, and cAMP in human cell lines. *J. Cell. Biol.* 106: 2159-2170
- Frizell, E., Abraham, A., Doolittle, M., Bashey, R., Kresina, T., Thiel, D. V. and Zern M. A. (1994) FK506 enhances fibrogenesis *in vitro* and *in vivo* models of liver fibrosis in rats. *Gastroenterology* 107: 492-498
- Fruman, D. A., Burakoff, S. J. and Bierer, B. E. (1994) Immunophilins in protein folding and immunosuppression. *FASEB J.* 8: 391-400
- Hojo, M., Morimoto, T., Maluccio, M., Asano, T., Morimoto, K., Lagman, M., Shimbo, T., and Suthanthiran, M. (1999) Cyclosporin induces cancer progression by a cell-autonomous mechanism. *Nature* 397: 530-534
- Lee, B.-H., Park, R.-W., Choi, J.-Y., Ryoo, H.-M., Sohn, K.-Y. and Kim I.-S. (1996) Stimulation of fibronectin synthesis through the protein kinase C signaling pathway in normal and transformed human lung fibroblasts. *Biochem. Mol. Biol. Int.* 39: 895-904
- Lee, B.-H., Park, R.-W., Sohn, K.-Y. and Kim, I.-S. (1997) Antagonistic regulation of protein kinase C-induced stimulation of fibronectin synthesis by cyclic AMP in human lung fibroblasts. *Biochem. Mol. Biol. Int.* 43: 383-390

- Lee, B.-H., Park, R.-W. and Kim, I.-S. (1998) Regulation of fibronectin gene expression by cyclic AMP and phorbol myristate acetate in HT-1080 human fibrosarcoma cells. *Exp. Mol. Med.* 30: 240-245
- Liu, J., Farmer, J. D. Jr., Lane, W. S., Friedman, J., Weisman, I. and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66: 807-815
- Myers, B. D. (1996) Cyclosporin nephrotoxicity. *Kidney Int.* 30: 964-974
- Norton, P. A. and Hynes, R. O. (1987) Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. *Mol. Cell Biol.* 7: 4297-4307
- Ruoslahti, E. (1988) Fibronectin and its receptors. *Ann. Rev. Biochem.* 57: 375-413
- Ruoslahti, E., Engvall, E. and Hayman, E. G. (1981) Fibronectin: current concepts of its structure and function. *Collagen Res.* 1: 95-128
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. 2nd ed., pp7.1-7.52, Cold Spring Harbor Laboratory Press, New York
- Schwarzbauer, J. E., Patel, R. S., Fonda, D. and Hynes, R. O. (1987) Multiple sites of alternative splicing of the rat fibronectin gene transcript. *EMBO J.* 6: 2573-2580
- Senger, D. R., Destree, A. T. and Hynes, R. O. (1983) Complex regulation of fibronectin synthesis by cells in culture. *Am. J. Physiol.* 245: C144-C150
- Wolf, G., Killen, P. D. and Neilson, E. G. (1990) Cyclosporin A stimulates transcription and procollagen secretion in tubulointerstitial fibroblasts and proximal tubular cells. *J. Am. Soc. Nephrol.* 1: 918-922
- Zardi, L., Camemolla, B., Siri, A., Petersen, T. E., Paoletta, G., Sebastio, G. and Baralle, F. E. (1987) Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously un-observed exon. *EMBO J.* 6: 2337-2342