

Received 11 May 2004
Accepted 9 June 2004

BIGLYCAN IS INTERNALIZED VIA A CHLORPROMAZINE-SENSITIVE ROUTE

MARTIN GÖTTE^{1,2,*}, DAVID DENIS SOFEU FEUGAING¹
and HANS KRESSE^{1†}

¹Department of Physiological Chemistry and ²Department of Obstetrics and Gynecology, Münster University Hospital, Domagkstr. 11, D-48149 Münster, Germany

Abstract: The small leucine-rich proteoglycan biglycan (BGN) is abundantly expressed in mesenchymal tissues. Its expression level is related to the phenotypic differentiation of cells. A dysregulation in BGN expression occurs under several pathological conditions, including glomerulonephritis, mesothelioma, pancreatic cancer and a mouse model of osteoporosis. Since the extracellular concentration of BGN is regulated both by secretion and endocytosis, we performed mechanistic studies on BGN endocytosis in human skin fibroblasts *in vitro*, using inhibitors of different endocytic routes. Chlorpromazine, an inhibitor of the clathrin-coated pit-pathway reduced endocytosis of BGN in human skin fibroblasts by 40%, and decreased degradation of BGN by 66%. Filipin, an inhibitor of the caveolae pathway, and Tyrphostin AG 1478, a specific inhibitor of EGF-receptor phosphorylation that partially inhibits endocytosis of the structurally related proteoglycan decorin, had no influence on BGN internalization and degradation. Our data indicates that the classical clathrin-mediated endocytic pathway is a major route for the internalization of BGN. Based on the differential susceptibility to pharmacological inhibition, it appears that BGN endocytosis seems to be at least in part mechanistically different from decorin uptake.

Key Words: Dermatan Sulfate, Endocytosis, Proteoglycan

*Corresponding author; tel: (+49) 251 8356113, fax: (+49) 251 8356114, e-mail: mgotte@uni-muenster.de

†Deceased

Abbreviations used: EGF – epidermal growth factor, SLRP – small leucine rich proteoglycan, TGF-β – transforming growth factor beta.

INTRODUCTION

Biglycan is a member of the family of small leucine rich proteoglycans (SLRP) [1, 2]. It is characterized by a 38 kDa core protein containing ten leucine-rich repeats flanked by disulfide bond stabilized loops, and it is substituted with several *N*-linked oligosaccharides and two glycosaminoglycan chains of the chondroitin-/dermatan sulfate type [1]. Biglycan is found at the cell surface or in the pericellular space in various tissues of mainly mesenchymal origin. Biglycan expression has also been associated with a variation in the phenotypic differentiation of cells; e.g. benign fibroblastic mesothelial cells express more biglycan than mesothelial cells with epithelial-like morphology [3]. Biglycan interacts with several collagens and regulates the activity of transforming growth factor beta (TGF- β) [1]. A major physiological role for biglycan in bone formation was revealed using biglycan knockout (*bgn*) mice, which display a reduced bone mass and an osteoporosis-like phenotype. These mice have a diminished capacity to produce bone marrow stromal cells, which are further characterized by a reduced response to TGF- β , reduced collagen synthesis and increased apoptosis [2]. Nielsen *et al.* [4] observed that, in contrast to wild-type mice, female *bgn* mice were resistant to trabecular bone loss as a consequence of estrogen depletion by ovariectomy. A dysregulation of biglycan expression is observed not only in the mouse model of osteoporosis, but also in additional pathological conditions such as glomerulonephritis, pancreatic cancer and mesotheliomas [5-8]. Since the amount of extracellular biglycan is not only regulated at the biosynthetic level, but also by endocytosis and subsequent lysosomal degradation [8, 9] it is important to understand the molecular mechanisms of biglycan uptake and degradation. Biglycan is efficiently internalized by receptor-mediated endocytosis in human skin fibroblasts, articular chondrocytes and rat mesangial cells [8, 9], but so far, very little is known about the endocytic route utilized by biglycan. In this study, we used selective pharmacological inhibitors of different endocytic routes in an established *in vitro* assay of biglycan endocytosis to gain new mechanistic insights into the biglycan endocytic pathway.

MATERIALS AND METHODS

Cell culture

Human skin fibroblasts [10] and CHO-K1 cells (chinese hamster ovary cell line) were maintained in modified Eagle's medium essential medium containing 10% fetal calf serum (FCS), non-essential amino acids, and antibiotics, as described previously [10]. HBL-100 cells (transformed human breast epithelial cell line) [11] were cultured in McCoy's 5A/10% FCS/2mM L-glutamine. CO60 cells (SV40-transformed chinese hamster embryo cell line) [12] were grown in DMEM/10% FCS. For endocytosis experiments, the concentration of NaHCO₃ was reduced from 2.2 g/l to 1.6 g/l to maintain a pH of 7.2 in an atmosphere of 95% air/5% CO₂.

Identification of biglycan-binding proteins

Biglycan was prepared under non-denaturing conditions by ammonium sulfate precipitation and anion-exchange chromatography on a Bio-Gel TSK DEAE-5PW HPLC column (Bio-Rad Laboratories, Munich, Germany) exactly as described previously [8]. Biglycan-binding proteins were identified using previously described procedures [9, 10].

Endocytosis assay

The rate of endocytosis of [³⁵S]sulfate-labelled biglycan was measured and analyzed exactly as described previously [8, 9]. Briefly, endocytosis is followed by the intralysosomal formation of inorganic sulfate, the major part of which is released into the culture medium. The level of endocytosis is represented by the sum of the level of intracellular radioactivity and the level of extracellular ethanol-soluble radioactivity. The rate of endocytosis is expressed as clearance rate, giving the volume of the incubation medium (in μ l) cleared from labelled biglycan per h and mg of cell protein [9]. For inhibitor studies, the respective inhibitors were used during a 1-h preincubation period, followed by a 3-h incubation with biglycan in the presence of the drug at the following concentrations [13]: Chlorpromazine (Sigma, Deisenhofen, Germany): 10 μ g/ml; filipin (Sigma): 3 μ g/ml; Tyrphostin AG1478 (Calbiochem, Darmstadt, Germany): 10 μ M. The results are expressed as the mean \pm SD (n=4). The data was analyzed using the paired Student's t-test. Differences were considered statistically significant at $P < 0.005$.

RESULTS AND DISCUSSION

Biglycan is efficiently endocytosed by human skin fibroblasts, articular chondrocytes and rat mesangial cells [8, 9]. Biglycan purified from the secretions of osteosarcoma cells interacts with putative receptor proteins of 51 and 26 kDa [9], which are found both at the cell surface and in endosomes. These proteins are also recognized by the SLRP decorin, which can compete for biglycan receptor binding [8, 9]. In this study, we used biglycan purified from transfected human embryonic kidney 293 cells [8]. To confirm the binding of this recombinant biglycan to the putative receptor proteins, we incubated [³⁵S]sulfate-labelled biglycan with blots of whole-cell extracts of human skin fibroblasts, as previously described for biglycan from osteosarcoma cells [9] and for decorin [10]. Since biglycan expression is differentially regulated in a variety of tumors [5-7], we also tested biglycan binding to cell extracts of two transformed cell lines, HBL-100 [11] and CO60 [12], including CHO-K1 cells as a control cell line (Fig. 1). Like biglycan from osteosarcoma cells [9], recombinant biglycan binds to fibroblast proteins of 51 and 26 kDa. A similar binding pattern is seen in the case of CHO-K1, CO60, and HBL-100 cells; however, the transformed cell lines have a higher capacity for biglycan binding.

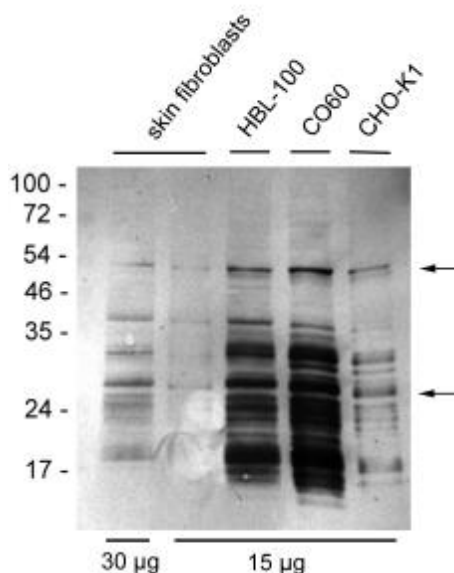


Fig. 1. Biglycan-binding proteins in cell extracts of normal and transformed cell lines. Whole cell extracts (15 μ g or 30 μ g of protein, respectively) of human skin fibroblasts, transformed human HBL-100 mammary epithelial cells, the SV40-transformed chinese hamster embryo cell line CO60 and chinese hamster ovary cells (CHO-K1) were separated by SDS-PAGE. After blotting and incubation with 650,000 cpm/ml [35 S]sulfate-labelled biglycan, the bound ligand was visualized via autoradiography. The migration distance of M_r standards (given as $10^{-3} \times M_r$) is shown in the left margin. Arrows indicate the previously described 51- and 26-kDa proteins [9], respectively.

In addition to the endocytosis receptor proteins, several additional biglycan-binding proteins could be detected in the whole-cell extracts. These proteins, including the most prominent species of about 30 and 14 kDa, had been described before [9]; however, they were not sensitive to trypsin treatment of intact cells and were not considered receptor proteins at the cell membrane.

We next used an established *in vitro*-system [9] to study the endocytosis of [35 S]sulfate-labelled biglycan in human skin fibroblasts in the presence of inhibitors of selective endocytic routes (Fig. 2). Chlorpromazine, a cationic amphiphilic drug that inhibits the assembly of the clathrin adapter protein AP2 on clathrin-coated pits [14], inhibited the endocytosis of biglycan in human skin fibroblasts by 40% (Fig. 2A). In addition, the degradation of biglycan was reduced by 66% in chlorpromazine-treated cells (Fig. 2B). This observation would be in accordance with an inhibition of receptor recycling by chlorpromazine, which was previously demonstrated for the LDL-receptor [14]. We recently observed a similar inhibition of decorin endocytosis by chlorpromazine under identical experimental conditions [13]. In addition, decorin endocytosis was significantly decreased by Tyrphostin AG1478,

a specific inhibitor of EGF-receptor phosphorylation [13]. However, this drug did not influence biglycan endocytosis (Fig. 2). We also tested filipin's effect on biglycan uptake and degradation; this cholesterol-depleting drug is commonly used to block endocytosis through caveolae [15] and had previously been shown to partially inhibit decorin uptake [13].

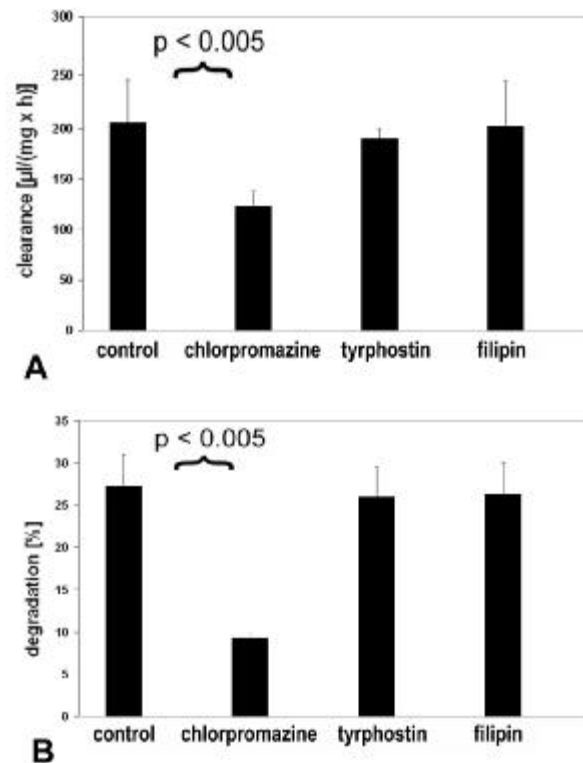


Fig. 2. The inhibition of biglycan endocytosis and degradation by chlorpromazine. Human skin fibroblasts in monolayer culture were preincubated for 1 h with or without 10 $\mu\text{g}/\text{ml}$ chlorpromazine, 10 μM tyrphostin AG1478, or 3 $\mu\text{g}/\text{ml}$ filipin, followed by a 3h incubation with 250,000 cpm/ml of [^{35}S]sulfate-labelled biglycan in the presence of the drugs. Chlorpromazine treatment affects both clearance (A) and degradation (B) of biglycan endocytosed by skin fibroblasts.

However, under identical experimental conditions, filipin had no effect on biglycan uptake and degradation (Fig. 2). Our data indicates that a major part of biglycan is endocytosed via the classical clathrin-coated pit mediated pathway. Both biglycan and the structurally highly related SLRP decorin are recognized by the same receptor proteins [9, 10]. However, it appears that biglycan endocytosis is at least in part mechanistically different from decorin uptake, based on the differential susceptibility to pharmacological inhibition of

endocytosis. Our study is a first step towards the mechanistic understanding of biglycan endocytosis, and paves the way for a pharmacological approach to pathological conditions involving biglycan dysregulation, such as osteoporosis, glomerulonephritis, mesothelioma and pancreatic cancer.

Acknowledgements. This study was supported by DFG SFB492/A6. The authors would like to thank Doris Holtfrerich and Tanja Terhörst for their expert technical assistance. M.G. and D.D.S.F. contributed equally to this work.

REFERENCES

1. Fisher, L.W. Biglycan (BGN). In: **Guidebook to the Extracellular Matrix, Anchor and Adhesion Proteins** (Kreis, T. and Vale, R. Eds) 2nd edition, New York: Sambrook & Tooze at Oxford University Press, 1999, 365-368.
2. Young, M.F., Bi, Y., Ameye, L. and Chen, X.D. Biglycan knockout mice: new models for musculoskeletal diseases. **Glycoconj. J.** 19 (2002) 257-262.
3. Gulyas, M., Dobra, K. and Hjerpe, A. Expression of genes coding for proteoglycans and Wilms' tumour susceptibility gene 1 (WT1) by variously differentiated benign human mesothelial cells. **Differentiation** 65 (1999) 89-96.
4. Nielsen, K.L., Allen, M.R., Bloomfield, S.A., Andersen, T.L., Chen, X.D., Poulsen, H.S., Young, M.F. and Heegaard, A.M. Biglycan deficiency interferes with ovariectomy-induced bone loss. **J. Bone Miner. Res.** 18 (2003) 2152-2158.
5. Weber, C.K., Sommer, G., Michl, P., Fensterer, H., Weimer, M., Gansauge, F., Leder, G., Adler, G. and Gress, T.M. Biglycan is overexpressed in pancreatic cancer and induces G1-arrest in pancreatic cancer cell lines. **Gastroenterology** 121 (2001) 657-667.
6. Chen, W.B., Lenschow, W., Tiede, K., Fischer, J.W., Kalthoff, H. and Ungefroren, H. Smad4/DPC4-dependent regulation of biglycan gene expression by transforming growth factor-beta in pancreatic tumor cells. **J. Biol. Chem.** 277 (2002) 36118-36128.
7. Gulyas, M. and Hjerpe, A. Proteoglycans and WT1 as markers for distinguishing adenocarcinoma, epithelioid mesothelioma, and benign mesothelium. **J. Pathol.** 199 (2003) 479-487.
8. Schaefer, L., Hausser, H., Altenburger, M., Ugorcakova, J., August, C., Fisher, L.W., Schaefer, R.M. and Kresse, H. Decorin, biglycan and their endocytosis receptor in rat renal cortex. **Kidney Int.** 54 (1998) 1529-1541.
9. Hausser, H., Ober, B., Quentin-Hoffmann, E., Schmidt, B. and Kresse, H. Endocytosis of different members of the small chondroitin/dermatan sulfate proteoglycan family. **J. Biol. Chem.** 267 (1992) 11559-11564.
10. Götte, M., Kresse, H. and Hausser, H. Endocytosis of decorin by bovine aortic endothelial cells. **Eur. J. Cell Biol.** 66 (1995) 226-233.
11. Caron de Fromentel, C., Nardeux, P.C., Soussi, T., Lavialle, C., Estrade, S., Carloni, G., Chandrasekaran, K. and Cassingena, R. Epithelial HBL-100 cell

- line derived from milk of an apparently healthy woman harbours SV40 genetic information. **Exp. Cell Res.** 160 (1985) 83-94.
12. Vanhorick, M. and Moens, W. Carcinogen-mediated induction of SV40 DNA amplification is enhanced by acrylamide in Chinese hamster CO60 cells. **Carcinogenesis** 4 (1983) 1459-1463.
 13. Sofeu Feugaing, D.D., Götte, M., De la Motte, C., Drazba, J., Kresse, H. Involvement of different uptake routes in decorin endocytosis. **Mol. Biol. Cell** 13 (2002) S1919.
 14. Wang, L.H., Rothberg, K.G. and Anderson, R.G. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. **J. Cell Biol.** 123 (1993) 1107-1117.
 15. Schnitzer, J.E., Oh, P., Pinney, E. and Allard, J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. **J. Cell Biol.** 127 (1994) 1217-1232.