Amino Acids (2008) 35:731–738
DOI 10.1007/s00726-008-0051-8

REVIEW ARTICLE

Prolidase-dependent regulation of collagen biosynthesis

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Received: 25 November 2007 / Accepted: 7 February 2008 / Published online: 5 March 2008

Abstract Prolidase [EC.3.4.13.9] is a cytosolic imidodipeptidase, which specifically splits imidodipeptides with C-terminal proline or hydroxyproline. The enzyme plays an important role in the recycling of proline from imidodipeptides (mostly derived from degradation products of collagen) for resynthesis of collagen and other proline-containing proteins. The enzyme activity is up-regulated by β_1-integrin receptor stimulation. The increase in the enzyme activity is due to its phosphorylation on serine/threonine residues. Collagen is not only structural component of extracellular matrix. It has been recognized as a ligand for integrin receptors, which play an important role in signaling that regulate ion transport, lipid metabolism, kinase activation and gene expression. Therefore, changes in the quantity, structure and distribution of collagens in tissues may affect cell signaling, metabolism and function. Several line of evidence suggests that prolidase activity may be a step-limiting factor in the regulation of collagen biosynthesis. It has been shown in different physiologic and pathologic conditions. It is of great importance during wound healing, inflammation, aging, tissue fibrosis and possibly skeletal abnormalities seen in Osteogenesis Imperfecta. The mechanism of prolidase-dependent regulation of collagen biosynthesis was found at both transcriptional and post-transcriptional levels. In this study, we provide evidence for prolidase-dependent transcriptional regulation of collagen biosynthesis. The mechanism was found at the level of NF-kB, known inhibitor of type I collagen gene expression. Modulation of integrin-dependent signaling by stimulatory (i.e. thrombin) or inhibitory (i.e. echistatin) β_1-integrin ligands or by nitric oxide donors (i.e. DETA/NO) affect prolidase at post-transcriptional level. All those factors may represent novel approach to pharmacotherapy of connective tissue disorders.

Keywords Prolidase · Collagen metabolism · β_1-integrin signaling

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Introduction

Prolidase (E.C.3.4.13.9) is a cytosolic exopeptidase widely distributed in man and animals (Myara et al. 1984). The enzyme cleaves imidodi- and imidotripeptides with C-terminal proline or hydroxyproline (Mock et al. 1990). The reaction catalyzed by prolidase is presented on Fig. 1. The best substrate for the prolidase and simultaneously the most abundant imidodipeptide is glycyl-proline (Mock et al. 1990). The imidodipeptides come from intracellular degradation of procollagen (intracellular form of collagen), collagen (extracellular form of collagen), degradation of other proline-containing proteins (sources representing very small percentage of imidodipeptides) and dietary proteins (Adibi and Mercer 1973; Jackson et al. 1975). Intestinal hydrolases do not recognize the inherent tertiary amide bonds (Myara et al. 1984), therefore, termination of ingested protein degradation take place intracellularly. It seems that prolidase activity (despite the collagen gene expression) may be a step-limiting factor in regulation of collagen biosynthesis. Cytosolic location of the

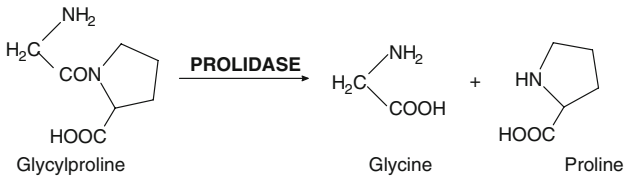

Fig. 1 Reaction catalyzed by prolidase

exopeptidase and its substrate specificity suggest that it may play an important regulatory role in the metabolism of proteins containing proline or hydroxyproline.

Prolidase gene and chromosomal localization

The prolidase gene (PEPD) has been isolated from cDNA library of human liver (Endo et al. 1987a; Endo et al. 1989; Wang et al. 2006) and placenta (Endo et al. 1989). The genomic sequence covers over 130 kilobases of genomic DNA, containing 15 exons encoding 492 amino acids. The coding sequences represent only 2% of the genomic sequences (Tanoue et al. 1990). Human prolidase gene was localized on chromosome 19 (Ball et al. 1991), on the short arm region (19p13.2). Structure of prolidase gene, its mRNA, subunit and native dimeric form of the enzyme is presented on Fig. 2.

Structure and physicochemical properties of prolidase

Prolidase, known also as Peptidase D or Iminopeptidase has been found in various mammalian tissues (Myara et al. 1984) and also in microorganisms (Barua et al. 1989; Lupi et al. 2006a). The enzyme was purified from several sources including human erythrocytes (Endo et al. 1987b), fibroblasts (Boright et al. 1989; Endo et al. 1989) and different strains of bacteria (e.g. *Escherichia coli*) (Lupi et al. 2006a). The human enzyme is homodimeric, the subunits having molecular weight 56–58 kDa (Boright et al. 1989; Richter

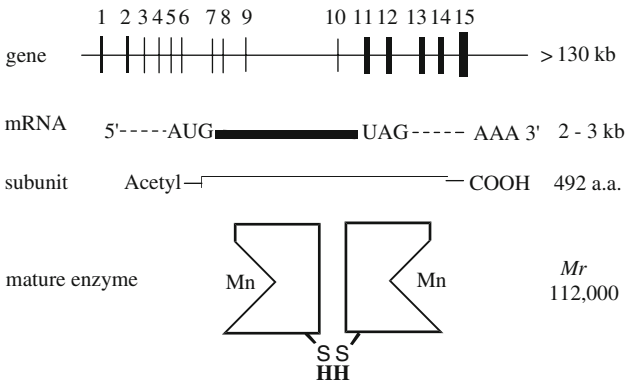

Fig. 2 Structures of the prolidase gene (PEPD), messenger RNA, subunit protein and the mature protein (according to Endo and Matsuda 1991), *a.a.* amino acids, *Mr* molecular range, *kb* kilo bases

et al. 1989). The molecular weight of native enzyme, estimated on SDS-PAGE is 97 kDa (Endo et al. 1988). The subunits from other mammalian tissues have masses similar to human enzyme but their native forms (homodimers) show marked variations in the range between 108 and 116 kDa (Myara et al. 1984). In case of some microorganisms, the enzyme is monomer of about 42 kDa (e.g., *Lactobacillus lactis*) or homodimer of about 80 kDa (e.g., *Pyrococcus furiosus*) (Ghosh et al. 1998). The primary structure of the human subunit has been deduced from the nucleotide sequence of cDNA clones of human erythrocytes. The polypeptide was found to contain 492 amino acid residues corresponding to molecular weight 54.3 kDa (Endo et al. 1989). During the process of subunit maturation the polypeptide chain is processed at the amino terminus by removal of methionyl residue and blocking the N-terminal alanine presumably by acetyl group. Prolidase is a glycoprotein containing about a half percent carbohydrate by weight (Sjostrom and Noren 1991). The secondary structure of human prolidase subunit has α-helices (36%, 20 helices, 181 residues) and β-sheet (16%, 32 strands, 81 residues) as estimated from crystallographic analysis found in Protein Data Bank, with evenly distributed hydrophobic and hydrophilic regions (Endo et al. 1989). Although the primary sequence of prolidase is unique, there are some sequences similar to those in certain regions of F1-ATPase and aminopeptidase P (Endo et al. 1989). Prolidase is metalloprotease. It has a specific requirement for manganese and the ion (Mn^{+2}) activates the enzyme (Mock and Green 1991). Native enzyme requires prolonged incubation with manganese at 37°C for maximum activity in vitro and is stable thereafter (Myara et al. 1984). The pH optimum of native enzyme is 7.6–7.8 and the isoelectric point is at pH 4.5 as determined by isoelectric focusing, implying a preponderance of acidic amino acids in its structure. Probably, arginine and anionic amino acid (glutamate/aspartate) residues are involved at the active site (Mock et al. 1990; Mock and Zhuang 1991; Myara et al. 1984). It has been found that cleavage activity involves deprotonation of a functional group of the polypeptide subunit (Mock and Green 1991). Studies with agents that block thiol residues (4-chloromercuribenzoic acid) imply that a cysteine residue is necessary for prolidase activity (Sjostrom 1974). The enzyme activity was found to be regulated by different sulfur amino acids (Uramatsu et al. 2007). One of the most effective prolidase inhibitor in vitro is *N*-benzyloxycarbonyl-L-proline (Lupi et al. 2005).

Substrate specificity for prolidase

Prolidase from various sources hydrolyze dipeptides in which the C-terminal amino acid proline or hydroxyproline

is linked through its tertiary nitrogen to the carbonyl of N-terminal amino acid residue (Mock et al. 1990).

Only the trans isomer of X-Pro dipeptides where X is any amino acid is cleaved by the enzyme (King et al. 1986; Lin and Brandts 1979). In respect to proline in X-Pro dipeptide, the L-configuration is essential (Sjostrom et al. 1973). Replacement of proline by hydroxyproline or sarcosine results in 50-fold loss in hydrolytic efficiency, whereas replacement of proline with thiazolidinecarboxylic acid leads to a substrate that is about three times more efficiently hydrolyzed than glycyl-proline (Hui and Lajtha 1978; Sjostrom et al. 1973).

Kinetic study of these substrates led to the conclusion that a functional group with a pK_a value of 6.6 is essential to the hydrolytic mechanism. The authors proposed that the group is water molecule rendered acidic by coordination to the metalloenzyme complex. The water molecule is displaced from the active site metal ion by the substrate, forming enzyme–substrate complex (Mock et al. 1990). A model for the enzyme–substrate complex has been proposed in which the active site Mn^{+2} cation is simultaneously ligated to the prolyl carboxyl group and the amido oxygen of the preceding residue of the *trans* X-Pro dipeptides (King et al. 1989). The scheme of the hypothetical complex is presented in Fig. 3. The highest susceptibility to the action of prolidase evoke alanyl-proline, glycyl-proline, valyl-proline and several fold lower than other imidodipeptides (Mock et al. 1990).

Recent data show that prolidase may also inactivate organophosphorous compounds (acetylcholinesterase-inhibiting drugs) suggesting that prolidase evokes broader substrate specificity than that was initially thought (Wang et al. 2006).

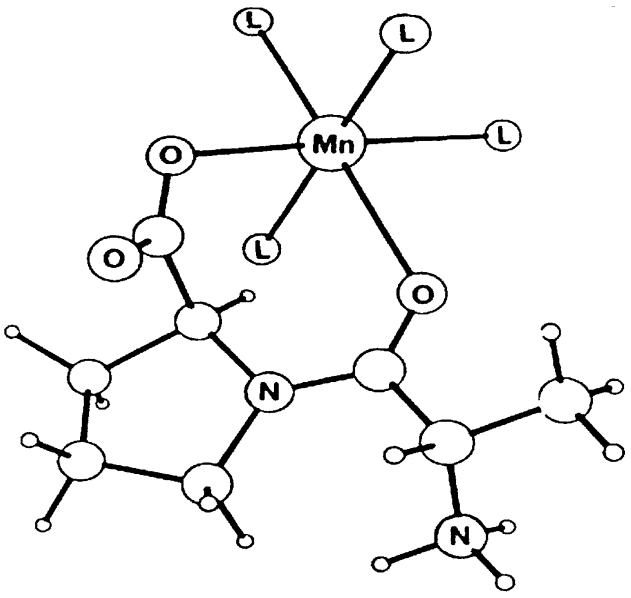

Fig. 3 Hypothetical model of prolidase–substrate (alanylproline) interaction (according to King et al. 1989). *N* nitrogen, *O* oxygen, *L* regions of prolidase binding manganese

Prolidase deficiency

Prolidase deficiency is rare autosomal recessive disorder characterized by massive imidodipeptiduria, skin lesions, recurrent infections, mental retardation and elevated proline-containing dipeptides in plasma (Freij et al. 1984; Goodman et al. 1968; Isemura et al. 1979; Pierard et al. 1984; Powell and Maniscalco 1976; Scriver 1964; Umemura 1978). The incidence of the disease is 1–2 cases per million births (Lemieux et al. 1984; Naughten et al. 1984, Lupi et al. 2006b). The estimate of disease prevalence will of course be low if symptomatic cases are not diagnosed as prolidase deficiency. On the other hand, some prolidase deficiency cases do not develop disease manifestations. Perhaps every person with prolidase deficiency will show symptoms if lifespan is sufficiently long but at present there is no explanation for the variation. The most characteristic symptoms of prolidase deficiency are connected to the metabolic changes in the connective tissue. All symptomatic cases had skin lesions as diffuse telangiectasia, purpuric rash, crasting erythematous dermatitis or progressive ulcerative dermatitis, particularly on the lower legs (Phang and Scriver 1989).

Mutations in prolidase gene are the molecular basis for prolidase deficiency. It has been found in several mutated alleles of prolidase gene. The mutations are present in exons 8, 10, 11, 12, 14 and 15, as well as in introns 4, 6, 7, 11 (Endo and Matsuda 1991; Ledoux et al. 1991; Lupi et al. 2006b). The products of mutated alleles are not able to form active (dimeric) enzyme (Tanoue et al. 1991) or undergo rapid degradation (Ledoux et al. 1996).

Role of prolidase in metabolism of collagen and other proline-containing proteins

The substrates (imidodipeptides) for prolidase come from intracellular degradation of procollagen, collagen and other proline or hydroxyproline-containing peptides, including dietary proteins. Prolidase catalyzes the final step of their degradation into free amino acids in the cytoplasm (Fig. 4). It seems that primary biological function of the enzyme in mammals involves the metabolism of collagen degradation products and the recycling of proline from X-Pro dipeptides for collagen resynthesis (Jackson et al. 1975; Yaron and Naider 1993). Therefore, in prolidase deficiency the connective tissue is affected, particularly its main component collagen. One of the clinical symptoms of the disease is skin lession. The pathogenetic mechanism leading to the

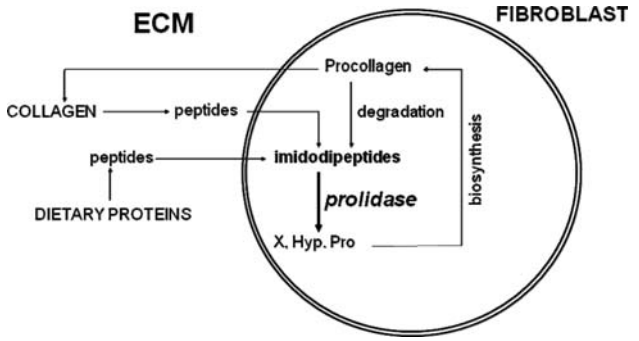

Fig. 4 Extracellular and intracellular sources of imidodipeptides (IDP), representing substrate for fibroblast's prolidase and its role in reutilization of proline for procollagen synthesis. *IDP* imidodipeptides, *CDP* collagen degradation products, *HYP* hydroxyproline, *Pro* proline, *X* any amino acid

skin changes is poorly understood. It is suggested that the pathomechanism of the disease is due to microangiopathy. Probably disturbances of collagen synthesis and degradation are an underlying process in which imidodipeptides (mainly glycyl-proline) may play an important role. It has been found that imidodipeptides stimulated intracellular collagen degradation. In fibroblast cultures from prolidase deficient patients, an increase in rapidly degraded collagen and decrease in proline pool has been found in comparison to control cells (Chamson et al. 1989). Collagen which accounts for about one-third of total body proteins represent polypeptide containing the highest amount of imino-bonds compared to all known proteins. In α1 subunit (1464 amino acids) of type I procollagen, proline is coupled to glycine 119 times and in α2 subunit (1366 amino acids) 106 times. In fully hydroxylated collagen, proline may occur either as hydroxylated amino acid in doublet of glycyl-hydroxyproline (gly-hyp) and unhydroxylated amino acid, glycyl-proline (Gly-pro). In α1 or α2 subunit of matured, hydroxylated collagen, Gly-pro may occur at least 25 times (Jackson et al. 1975). Therefore, degradation of collagen produces large quantities of Gly-pro, the substrate for prolidase.

Collagen is essential for the maintenance of connective tissue. The interaction between cells and ECM proteins, mediated by the integrin family of cell surface receptors can regulate cellular gene expression, differentiation, growth and other functions (Akiyama et al. 1990; Bissel 1981; Carey 1991; Donjacour and Cunha 1991). The functional link between collagen and prolidase activity was found in cultured human skin fibroblast treated with anti-inflammatory drugs (Miltyk et al. 1996), pyrroline 5-carboxylate (Miltyk and Palka 2000), during experimental aging of the cells (Palka et al. 1996), experimental inflammation in chondrocytes (Karna et al. 2006), cell surface integrin receptor ligation (Palka and Phang 1997), Osteogenesis Imperfecta (Galicka et al. 2001) and several malignant diseases (Karna et al. 2000, 2002; Cechowska et al. 2006).

Collagen is not the only protein in which proline is coupled to glycine. Similar sequence has been found in heavy chains of immunoglobulins and C_1q (Reid and Porter 1976). Since prolidase deficiency is accompanied by immunodeficiency (Phang and Scriver 1989) it cannot be excluded that the phenomenon is a result of disturbances in biosynthesis of immunoglobulin and C_1q. In view of the collagen-like amino acid sequence in both substances, it seems that there may be an immunologic deficit related to the prolidase deficiency.

In most cases of prolidase deficiency, mental retardation accompanies the disease (Phang and Scriver 1989). It may result from decrease in proline concentration in central nervous system. It has been found that proline play an important role in modulation of glutamatergic neurons (Fremeau et al. 1992). It is possible that modulation of prolidase activity may serve as a regulator of proline concentration in central nervous system. Not only brain prolidase may evoke such function. Prolidase of erythrocytes may cleave the dipeptides that penetrate the erythrocyte membrane and produce amino acids. Because the erythrocytes cannot utilize amino acids, it was suggested that the principal role of the amino acid transport system present in this cells is to efflux the amino acids from the hydrolysis of absorbed peptides (King and Kuchel 1984). In view of the facts, prolidase deficiency may be responsible for the decrease in proline pool in central nervous system and disturb the function of glutamatergic neurons.

Although the role of prolidase in pathobiochemistry of the disorders is not well understood, it seems that prolidase-dependent regulation of transcription factors activity may be an underlying mechanism. Recently, we have shown that overexpression of prolidase resulted in increased nuclear hypoxia inducible factor (HIF-1α) level and elevated expression of HIF-1 α dependent gene products, vascular endothelial growth factor (VEGF) and glucose transporter-1 (Glut-1). The activation of HIF-1-dependent transcription was shown by prolidase-dependent activation of hypoxia response element (HRE)-luciferase expression. We used an oxygen-dependent degradation domain (ODD)-luciferase reporter construct as a surrogate for HIF-1α as an in situ prolyl-hydroxylase assay. Since this reporter is degraded by VHL-dependent mechanisms, the increased levels of luciferase observed with prolidase expression reflected the decreased HIF-1α prolyl hydroxylase activity. Additionally, the differential expression of prolidase in two breast cancer cell lines showed prolidase-dependent differences in HIF-1α levels. These findings show that metabolism of imidodipeptides by prolidase plays a previously unrecognized role in angiogenic signaling (Surazynski et al. 2007).

Since prolidase serves a unique function in all cell types, therefore the mechanism(s) and factor(s) involved in prolidase activity regulation are of special interest.

Prolidase-dependent regulation of collagen biosynthesis

Collagen is known as a ligand for $\alpha 2\beta 1$ integrin. Previously, it has been shown that β_1-integrin receptor is involved in signaling that regulates collagen biosynthesis (Ivaska et al.1999) and prolidase activity (Palka and Phang 1997). Stimulated β_1-integrin receptor induces autophosphorylation of non-receptor focal adhesion kinase pp125FAK (FAK), which is then capable of interaction with adaptor proteins, such as Grb2, through Src or SHc proteins. This interaction allows to activate further cascade of signaling pathway through Sos, Ras and Raf proteins and subsequently two mitogen activated protein (MAP) kinases ERK1 and ERK2 (Juliano and Haskill 1993; Seger and Krebs 1995). The result of this phenomenon is induction of transcription factors that stimulate expression of genes for many proteins involved in the regulation of cell growth, differentiation and metabolism (Labat-Robert and Robert 2000). The evidence for the coordinate regulation of prolidase activity and collagen biosynthesis by β_1-integrin signaling comes from the study on the effect of echistatin (disintegrin) and thrombin (integrin activator) in human dermal fibroblasts. Echistatin was found to inhibit collagen biosynthesis and prolidase activity and expression, as well as FAK, Sos and MAPK expression, while thrombin in every case evoked an opposite effect (Surazynski et al. 2005a). Similarly, coordinate regulation of prolidase activity and collagen biosynthesis was found at the level of signaling by insulin-like growth factor (IGF-I), the most potent stimulator of collagen biosynthesis (Miltyk et al. 1998).

Studies on inhibition of prolidase activity by nickel chloride in CHO cells that are auxotrophic for proline, but contain prolidase showed that prolidase in these cells is important limiting factor for collagen production (Miltyk et al. 2005). The same correlation was found in case of stimulation of prolidase by nitric oxide (NO) donors. As shown in time course experiment, NO stimulate both prolidase activity and collagen biosynthesis in fibroblasts (Fig. 5). The mechanism for the stimulatory effect of NO on prolidase activity was found at the level of post-translational modification of prolidase. Increase in the enzyme activity was due to increase in the enzyme phosphorylation on serine/threonine residue (Surazynski et al. 2005b).

Several data suggested that prolidase-dependent regulation of collagen biosynthesis may take place at the transcriptional level. We provided evidence that transfection of colorectal cancer cells with prolidase vector

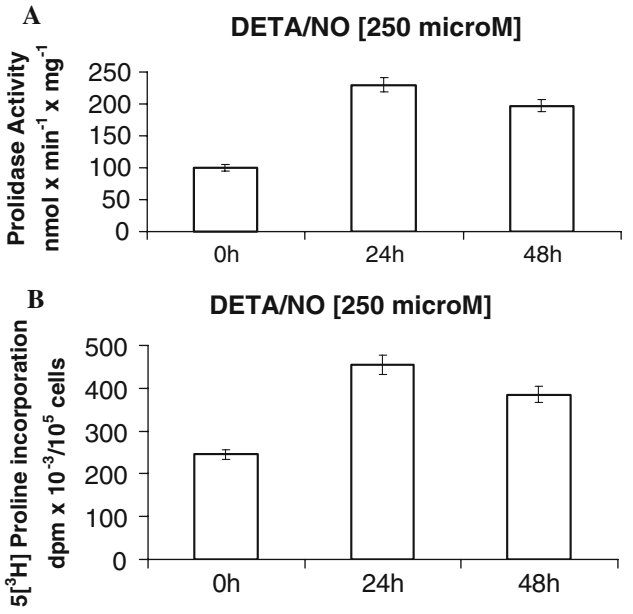

Fig. 5 Prolidase activity (**a**) and collagen biosynthesis (**b**) measured in NIH 3T3—fibroblast that had been treated with nitric oxide (NO) donor—DETA/NO at 250 μM for 24 and 48 h. Prolidase activity was measured according to the method of Myara et al. (1982) and collagen biosynthesis by the method of Peterkofsky et al. (1982)

drastically inhibited NF-kB expression (Fig. 6). The transcription factor is well-recognized inhibitor of expression of $\alpha 1$ and $\alpha 2$ subunits of type I collagen (Kouba et al. 1999; Rippe et al. 1999; Miltyk et al. 2007). Another evidence for the role of prolidase in regulation of NF-kB expression provides experiment showing that inhibition of prolidase

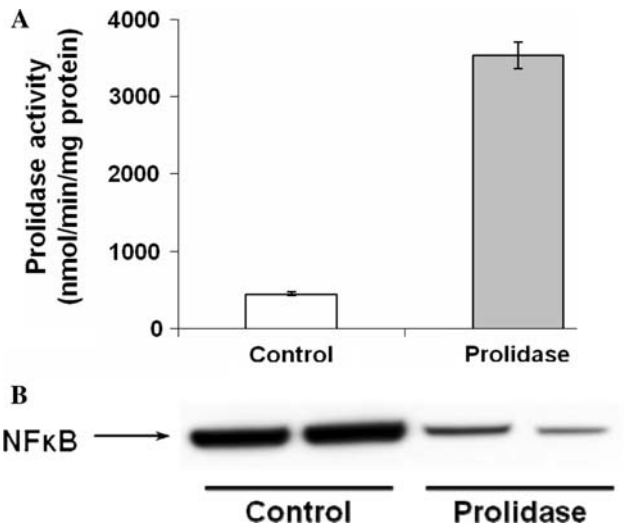

Fig. 6 Prolidase activity (**a**) and NF-kB expression (**b**) in cells with a gain-of-function experimental model by transfecting RKO cells with a prolidase cDNA expression plasmid and obtained stable transfectants. Prolidase activity was measured according to the method of Myara et al. (1982) and NF-kB expression by Western immunoblot

activity by Cbz-pro contributed to up-regulation of NF-kB expression in fibroblasts (Fig. 7).

Although the mechanism of this phenomenon is not known, it seems that products of prolidase activity, proline or hydroxyproline may modulate processing of NF-kB in a similar way as it was described with respect to HIF-1α (Surazynski et al. 2007). The activation of NF-kB requires degradation of the cytoplasmic IkBα by the ubiquitin–proteasome pathway. Increase in prolidase activity that result in increase in free proline or hydroxyproline may contribute to inhibition of degradation of IkBα and subsequently inhibition of NF-kB activity and translocation to the nucleus. However, the hypothesis requires to be proved. Whatever is the mechanism of the observed phenomena the data suggest that prolidase-dependent regulation of collagen biosynthesis may take place at both transcriptional and post-transcriptional levels.

Conclusions and perspectives

Although prolidase was found to play important role in the metabolism of various tissues most intriguing questions still remain unanswered: What factors are involved in prolidase activity regulation? What mechanisms explain the distant manifestation of the prolidase deficiency? What is the role of imidodipeptides in the metabolism of various tissues? What is the role of prolidase in pathology, including cancer?

Much of the work has to be done before the role of prolidase in metabolism of various tissues will be understood and before an effective treatment of prolidase deficiency will be developed.

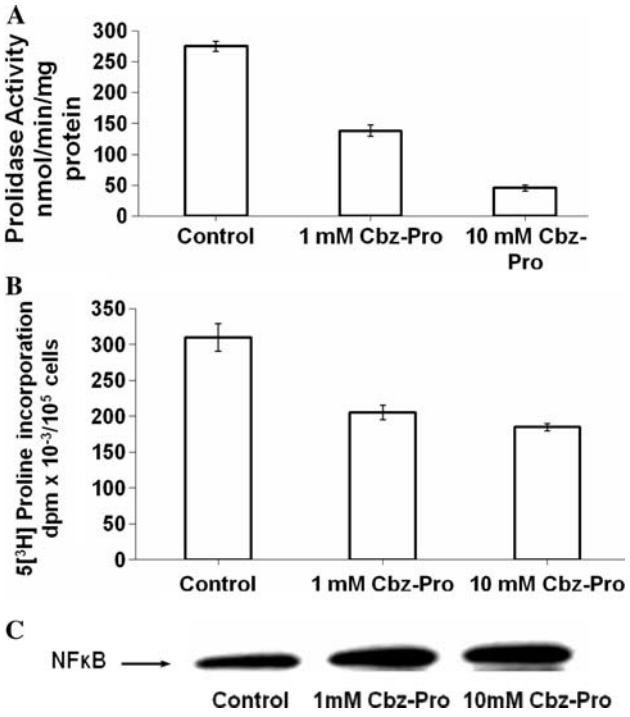

Fig. 7 Prolidase activity (**a**), collagen biosynthesis (**b**) and NF-kB expression (**c**) measured in fibroblast treated with different concentration of Cbz-Pro (prolidase inhibitor) for 24 h. The same methodology for the assays as in legend to Fig. 6 was employed

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