Synthesis and refolding of human TIMP-2 from *E. coli*, with specific activity for MMP-2

Alessandro Negro[a,*], Maurizio Onisto[b], Laura Masiero[b], Spiridione Garbisa[b]

[a]*Department of Biochemistry, School of Veterinary Medicine, University of Padova, Via Trieste 75, 35121 Padova, Italy*
[b]*Institute of Histology and General Embryology, University of Padova, Via Trieste 75, 35121 Padova, Italy*

Received 6 January 1995

Abstract Tissue inhibitors of metalloproteinase (TIMPs) are inhibitory counterparts of collagenases, containing 12 cysteine residues paired to six internal disulphide bridges. TIMP-2, an inhibitory protein of 72 kDa gelatinase/type IV collagenase (MMP-2), was expressed in *Escherichia coli* as a fusion protein with a 34 amino acid NH_2-linked tail containing six consecutive histidine residues. The protein was purified in a single-step using an ion metal affinity column (IMAC) in denaturing conditions. The immobilized fusion TIMP-2 protein was refolded at a high concentration in the column, producing about 5 mg of protein per litre of bacterial cells. It shows specific binding and inhibitory activity against MMP-2, but has no effect against 92 and 45 kDa gelatinases.

Key words: Tissue inhibitor of metalloproteinase; Type IV collagenase; Protein refolding; Ion metal affinity column

1. Introduction

TIMP-2, the second member of the family of tissue inhibitors of matrix metalloproteinases (MMPs) [1–3] is a non-glycosylated 21 kDa protein which exerts activity against several members of the MMP family, although preferentially toward the 72 kDa gelatinase/type IV collagenase (MMP-2 or gelatinase A), by binding to both its latent and activated forms [4,5]. MMP-2 expression has been repeatedly correlated to invasive and metastatic aggressiveness of tumour cells, and TIMP-2 inhibits matrix invasion by transformed cells [6] and endothelial cell proliferation [7] in vitro, as well as metastasis in vivo [8]. Nevertheless, deeper comprehension of proteinase–inhibitor interactions is still crucial for designing and checking pharmacological strategies for controlling pathological matrix proteolysis. Although the availability of TIMP-2 active protein from natural sources poses a serious limitation in this respect, DNA recombinant technology may help.

TIMP-1, the first cloned member of the tissue inhibitor family, has been produced more or less successfully by recombinant techniques using mammalian cells [9], *E. coli* [10–13] or baculovirus systems [14,15]. The second member, TIMP-2, was later produced only using mammalian cell systems [16–18].

Although DNA technology now permits burst synthesis of heterologous proteins in *E. coli*, these often accumulate as insoluble inclusion bodies, and denaturation–renaturation systems are necessary to obtain the native conformation [19].

Protein renaturation is not a simple process, above all when large and complex molecules are involved. The main problem is usually protein insolubility, as a consequence of the aggregation of incorrectly folded molecules, which in turn may be due to the presence of incorrect disulphide bridges. For this reason, most refolding systems involve dilution of denatured proteins to $\leq 10\ \mu M$ in an appropriate folding buffer. Several strategies have been developed to obtain renaturation of proteins in the presence of redox agents such as glutathione, β-mercaptoethanol, metal ions, denaturing agents, etc. Although a number of these systems do produce good results in some protein models, due to the multiple chemical–physical properties characteristic of each protein, yield is often poor and more appropriate refolding must be tailored.

We tackled this need in TIMP-2, which contains 12 cysteine residues linked through 6 disulphide bridges. Fused to a NH_2-tail of 34 amino acids, TIMP-2, was highly expressed in *E. coli*, the chimeric protein was isolated by affinity chromatography specific for the tail, and refolding was obtained with the protein still linked to the resin. The refolded recombinant fusion protein retains its specific binding and inhibitory activity for 72 kDa gelatinase/type IV collagenase.

2. Materials and methods

2.1. Materials

Restriction endonuclease and T4 DNA ligase were purchased from Gibco-BRL. Deoxyoligonucleotide primers were synthesized using phosphoramitide chemistry and an Applied Biosystem 380B automated DNA synthesizer. The polymerase chain reaction was performed using the Amplitaq kit (Cetus-Perkin Elmer). Plasmid pRSETA was purchased from Invitrogen. The host cell line *E. coli* BL21 (DE3) was generously provided by Dr. Stan Tabor (Harvard Medical School). DNA manipulation, transformation and plasmid purification were performed according to published procedures [20].

2.2. Construction of pSETIMP-2 plasmid

TIMP-2 gene from a pT2-MO1 plasmid-containing fragment [21] was cloned directly in pRSETB plasmid, which originated pRSETIMP-2 plasmid. The latter was constructed in *E. coli* strain HB101 and sequences were verified by double-strand dideoxy sequencing [22] using a sequenase reagent kit (United States Biochemical).

2.3. Synthesis and purification of recombinant protein

The purified pRSETIMP-2 expression plasmid was transferred into *E. coli* BL21 (DE3) for expression of the recombinant protein. Freshly prepared bacterial cells bearing the plasmid were grown at 37°C in 2 liters of Luria's Broth with 100 μg/ml ampicillin until the absorbence of the culture suspension reached 0.7 (590 nm). Expression of rhTIMP-2 was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside to inactivate the *lac* repressor, thereby allowing synthesis of T7 RNA polymerase which, in turn, transcribes DNA sequences next to the T7 promoter, with the subsequent production of the fusion protein [23]. Three hours after induction the bacteria were harvested by centrifugation, washed in phosphate-buffered saline (PBS) and resuspended in PBS. Cells were lysed by sonication (Branson Sonifer at power setting 6) for 5 min at 4°C in 6 M guanidinium-HCl, 0.1 M NaH_2PO_4 and

*Corresponding author. Fax: (39) (49) 8286-6659.

SSDI 0014-5793(95)00073-9

10 mM Tris-HCl (pH 8.0). The lysate was cleared by centrifugation at 12,000 × g for 20 min and then filtered through a 0.45 μm Millipore membrane. This filtrate was applied to an immobilized metal ion affinity chromatographic (IMAC) column (0.8 × 4 cm Ni^{2+}-NTA; Diagen). After exhaustive washing with 8 M urea, 0.1 M NaH_2PO_4 and 10 mM Tris-HCl (pH 8.0), the recombinant protein was eluted from the IMAC column with 8 M urea, 0.1 M NaH_2PO_4 and 10 mM Tris-HCl, using a pH 5.9–4.5 step gradient.

2.4. Refolding in solution of rhTIMP-2

IMAC-purified rhTIMP-2 (1 mg/ml) was diluted to 6 M urea and the pH was adjusted to 7.4. β-mercaptoethanol was then added to a final concentration of 140 mM, and the solution diluted 4 times in 6 M urea, 250 mM cystamine and 50 mM Tris-HCl (pH 7.4). The mixture was further diluted to 0.3 M urea by adding 50 mM Tris-HCl (pH 9.0), the final solution adjusted to pH 8.3, and kept for 24 h at 4°C. The diluted protein was concentrated 20 times by ultrafiltration on a YM10 Amicon membrane.

2.5. Solid-phase refolding of rhTIMP-2

Two mg of IMAC-purified rhTIMP-2 were reduced with 10 mM β-mercaptoethanol and loaded once again in a 2-ml IMAC resin column, in 100 mM Tris-HCl (pH 8.0) with continuous recycling. The resin was recovered and dialysed slowly against 1 M urea (pH 8.3) and, after three days at 10°C, the recombinant protein was eluted from it with 250 mM imidazole, 0.1 M NaH_2PO_4 and 10 mM Tris-HCl (pH 8). The fusion protein was then exhaustively dialysed against TIMP-buffer (20 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$ and 0.005% Triton X-100, pH 7.5). Protein concentration was determined by ultraviolet (UV) absorbent spectroscopy at 280 nm using a double-beam Perkin-Elmer spectrophotometer model Lambda-2 and 1-cm path length quartz cuvettes. An extinction coefficient of 0.1% (w/w) at 280 nm of TIMP-2 protein was taken as 1.46 $mg^{-1} \cdot cm^2$. Extinction coefficients were determined according to the method of Gill and von Hippel [24].

2.6. Binding of 72 kDa collagenase to rhTIMP-2

100 μg of refolded rhTIMP-2 were incubated for 2 h at room temperature with fresh medium and medium conditioned by A2058 cells. Subsequently, 200 μl of IMAC resin washed in H_20 were added to the medium, incubated for 2 h at room temperature, and packed into an EconoColum (BioRad). After exhaustive washing with phosphate-buffered saline (pH 7.8), the bound proteins were eluted from the column with 250 mM imidazole.

2.7. Analytical SDS-polyacrylamide gel electrophoresis and Western blot analysis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed using an acrylamide concentration of 5% in the stacking and 12% in the separating gel, stained with Coomassie brilliant blue. For Western blotting, after SDS-PAGE, samples were electro-transferred, overnight at 40 mA to HighBond-C extra nitrocellulose membranes (Amersham). The membranes were saturated for 1 h with 3% gelatin in Tris/Tween-20 buffer solution, then challenged with 1:500 rabbit antibodies against TIMP-2 [5] followed by 1:1000 anti-rabbit immunoperoxidase-conjugated Ab (BioRad 170-6515) and HRP color development reagent (BioRad).

2.8. Fluorescence spectroscopy

Fluorescence measurements were carried out on a Perkin-Elmer fluorescence spectrophotometer model LS-50 using a 0.5 ml internal volume quartz cell with a 1-cm path length, thermostatted at 25°C. Samples of rhTIMP-2 were prepared by dilution to 2 mM final concentration in 100 mM NaCl, 10 mM sodium phosphate buffer (pH 7.5) and, when specified, 6 M guanidine hydrochloride (GdnHCl). After excitation of samples at 280 nm, fluorescence emission spectra were recorded from 300 to 450 nm. Four consecutive accumulations were averaged after subtraction of the corresponding base-line.

2.9. Cell lines

HT1080 human fibrosarcoma cells, and two variant clones from Lewis lung carcinoma, C87-LLC and BC215-LLC [25] were grown in Dulbecco's modified Eagle's medium (Flow) supplemented with 10% heat-inactivated fetal calf serum, 25 μg/ml gentamycin (Sigma), in 5% CO_2 in air at 37°C. At 60–70% confluence, the cells were maintained in serum-free medium for additional 24 h, and the conditioned media were clarified and stored at −80°C until use.

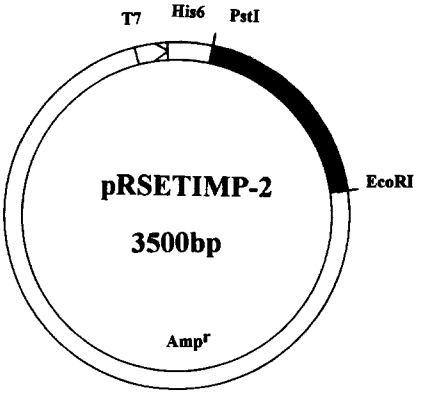
2.10. Gelatin zymography

Gelatinolytic activity of all conditioned media was assayed by a modification of Clark's method [26], by electrophoresing unheated and unreduced samples in 1 mg/ml gelatin containing polyacrylamide gels (BioRad). After removal of SDS by incubation in 2.5% (v/v) Triton X-100 for 30 min, the gels were incubated overnight at 37°C in 50 M Tris-HCl (pH 7.4) containing 0.2 M NaCl and 5 mM $CaCl_2$. The gels were stained for 30 min in 30% methanol/10% glacial acetic acid containing 0.5% (w/v) Coomassie brilliant blue R-250, and destained in the same solution without dye. Gelatinolytic activity was evident as clear bands against a blue background. Their M_r were estimated by comparison with low-range pre-stained SDS-PAGE standards (BioRad).

3. Results

The gene for TIMP-2 was cloned as a fusion protein directly in the commercially available vector pRSETA, under the control of T7 RNA polymerase-dependent promoter (Fig. 1). The recombinant vector pRSETIMP-2 coded for a 234 amino acid protein with a 34 amino acid tail at the TIMP-2 NH_2-terminal (Fig. 1, below) with six consecutive histidine residues, which were effective in mediating its binding to an IMAC column [27]. This tail region had an enterokinase cleavage site DDDDK for eventual cleavage of the fusion protein. Five glycine residues made this extra domain flexible, as predicted by the FLEXPRO program (PCGENE Software, release 6.8 Intelligenetics) (data not shown).

E. coli strain BL21 (DE3), containing the T7 RNA polymerase gene under the control of the *lac* promoter, was transformed with plasmid pRSETIMP-2 and induced with IPTG. About 5% of total bacterial proteins were represented by a molecule of 28 kDa M_r, which is in agreement with the molec-

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                    20                  40                  60
MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSELEICSCSPVHPQQAFCNADVVIR

                    80                  100                 120
AKAVSEKEVDSGNDIYGNPIKRIQYEIKQIKMFKGPEKDIEFIYTAPSSAVCGVSLDVGG

                    140                 160                 180
KKEYLIAGKAEGDGKMHITLCDFIVPWDTLSTTQKKSLNHRYQMGCECKITRCPMIPCYI

                    200                 220
SPDECLWMDWVTEKNINGHQAKFFACIKRSDGSCAWYRGAAPPKQEFLDIEDP
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Fig. 1. Structure of expression vector coding fusion protein His-TIMP-2. Note direction of T7 DNA polymerase dependent promoter and relative position of histidine residues in fusion protein. Below: nucleotide sequence of His-TIMP2 recombinant fusion protein.

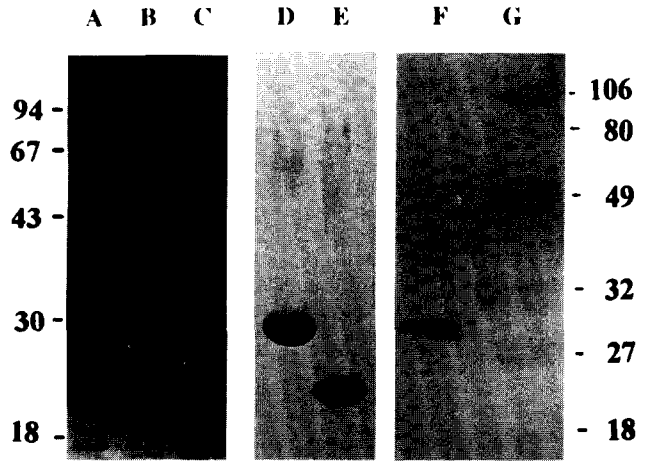

Fig. 2. Recombinant hTIMP-2 fusion protein expressed by *E. coli* and purified in IMAC column. Lane A = protein expressed by *E. coli* BL21 (DE3) transformed with plasmid pRSETIMP-2; lane B = eluted proteins from IMAC column at pH 5.9; lane C = eluted proteins from IMAC column at pH 4.5; lane D = refolded rhTIMP2 (reduced); lane E = refolded rhTIMP2 (unreduced); lane F = Western blot of rhTIMP2 with antibodies prepared against native human TIMP-2; lane G = prestained molecular weight markers (BioRad).

ular weight deduced from fusion protein DNA sequences (26,369 MW) (Fig. 2). The recombinant fusion protein, stored in *E. coli* as inclusion bodies, was directly solubilized by guanidinium and purified on an IMAC column [27].

The purified rhTIMP-2 was judged to be 95% pure by SDS-PAGE in the presence of β-mercaptoethanol and in denaturing conditions. In non-reducing conditions, it revealed the presence of multimer bands, thus indicating uncorrected folding: this was also highlighted by protein precipitation and loss of biological activity when the urea was dialysed out. The rhTIMP-2 was recognised as a unique 30 kDa band by antibodies prepared against native human TIMP-2, as demonstrated by Western blotting of reduced rhTIMP-2 (Fig. 2).

In order to obtain biologically active rhTIMP-2, we tried to refold rhTIMP-2 in the conditions used for rhTIMP-1 by Kohono et al. [11] in the presence of cystamine, although this procedure was reported as unsuccessful by Kleine et al. [13] for rhTIMP-1 and Cocuzzi et al. [12] for mouse TIMP-1. Kohono's procedure also failed to produce active human rTIMP-2 from *E. coli* (data not shown). A solid-phase refolding method was then optimised for TIMP-2, taking advantage of histidine binding to Ni^{2+} NTA resin. The procedure was carried on at a low protein/resin ratio (1 mg/ml resin), although this resin has a capacity of 10 mg protein per ml, as specified by the manufacturer. This system was effective in recovering rhTIMP-2 which, although the protein was eluted out of the column at a high concentration (1 mg/ml), remained soluble. SDS-PAGE analysis of the refolded rhTIMP-2 revealed only the lower approximately 26 kDa band, without a multimer ladder. The shift in M_r between reducing and non-reducing conditions fitted that observed for natural bovine TIMP-2, although the molecular weight of rhTIMP-2 was slightly higher, due to its extra tail.

Additional information on the tridimensional structure of the refolded rhTIMP-2 was inferred from fluorescence spectroscopy analysis. Fig. 3 shows that the l_{max} of the peak relative fluorescence of native and denatured rhTIMP-2 (in the absence or presence of 6 M guanidinium) is red-shifted from 339 to 352 nm. This is consistent with protein denaturation, in which the buried tryptophan residues are fully exposed to the solvent [28]. Moreover, the increase in fluorescence intensity of the denatured with respect to the native molecule indicates that the three tryptophan residues are quenched in the native structure [29].

The biological properties of recombinant hTIMP-2 were then checked by developing zymograms of tumour-secreted MMP-9 and MMP-2 gelatinases in buffer containing rhTIMP-2. As shown in Fig. 4, although rhTIMP-2 completely inhibited 72 kDa gelatinase, both as a zymogen and in the activated form, it did not inhibit any other gelatinolytic activity.

Binding to the protease and inhibitory activity involve two separate TIMP-2 domains, respectively at the COOH- and NH_2-terminal domains of the inhibitor [30–32]. We therefore verified whether rhTIMP-2 can bind other gelatinases. Addition of rhTIMP-2 and ion-metal affinity resin to gelatinases containing media resulted in selective recovery of MMP-2 alone, both as a zymogen and in its activated form (Fig. 4), with no other gelatinolytic activity.

4. Discussion

Recombinant technology usually has to use mammalian eukaryotic systems in order to produce active mammalian proteins. This is due mainly to the critical roles of both formation of correct disulphide bonds and glycosylation requirements.

Since TIMP-2, unlike TIMP-1, is a non-glycosylated protein, we optimised its expression using a prokaryotic system, which is less expensive and time-consuming, paying attention to the refolding process. This was because TIMP-2 contains 12 cysteine residues which have the potential to originate numerous disulphide bridges, and at high protein concentrations both

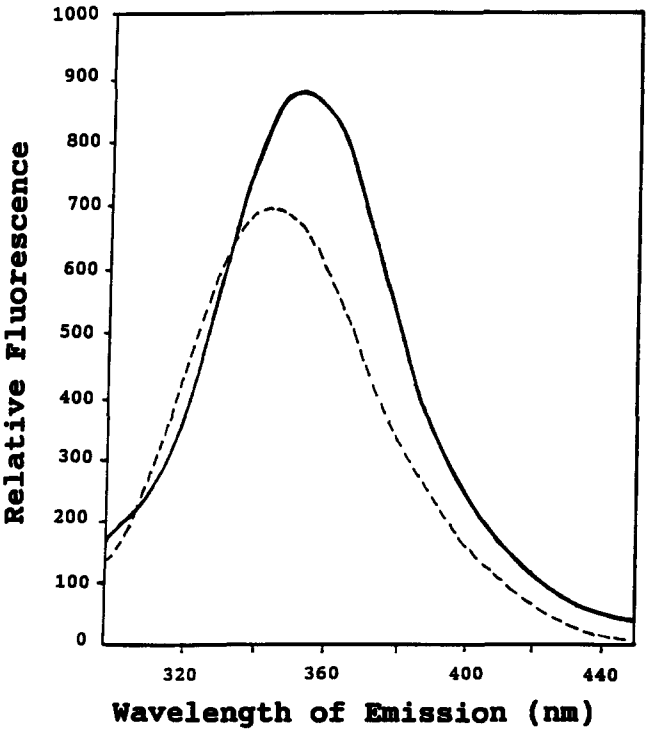

Fig. 3. Fluorescence emission spectra of refolded rhTIMP-2 in 0 M (dotted line) and 6 M (solid line) GdnHCl (λ_{ex} = 280 nm). In both cases, were used the concentration of 10 μg/ml of rhTIMP-2.

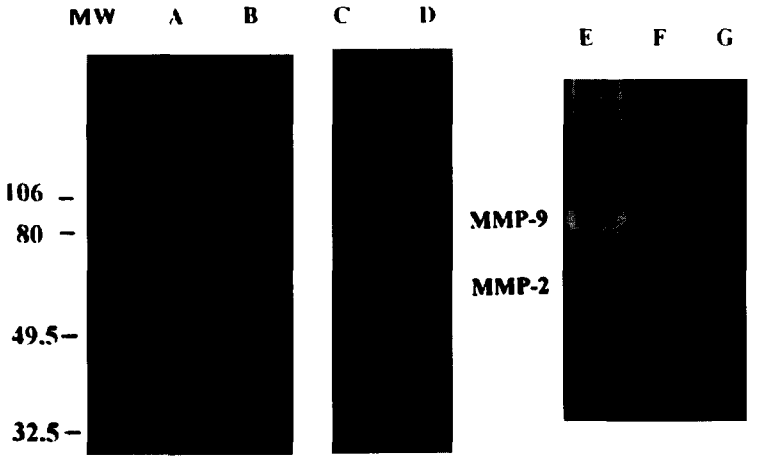

Fig. 4. Gelatin zymography of cells conditioned media. Lanes A and C = C87-LLC cells; lanes B and D = BC215-LLC cells; lanes C and D = were developed in presence of rhTIMP-2. In the right panel, specific affinity purification of MMP-2 from HT1080 cells by IMAC-bound rhTIMP-2: lane E = media conditioned by HT1080 cells; lane F = fresh medium; lane G = enzymatic activity after affinity chromathography and elution with imidazole.

intra- and inter-chain bonds may take place: in both cases, incorrect disulphide bonds may result. To avoid this problem, during refolding the protein must be diluted below 20 μg/ml, thus avoiding aggregation, precipitation and loss of activity [13]. Nevertheless, because of the intrinsic chemical–physical characteristics of each protein, satisfactory activity yield is not routinely achieved. In order to avoid aggregation, one important requirement is to maintain physical separation between the molecules before their correct refolding. This has been now achieved by immobilizing rhTIMP-2 on an affinity chromatographic resin, through a flexible, especially engineered tail. This extension gives the steric freedom necessary to reach the native conformation, with the progressive removal of reducing and denaturing agents. Particular attention was paid to this step for correct refolding of the molecule: while in the above procedures the reducing and denaturing agents were directly diluted by water addition, we preferred to slow down the process by dialysis.

Recombinant hTIMP-2 refolding was verified by a series of analyses. The higher M_r after SDS-PAGE of the molecule was indicative of compact folding; the change in fluorescence spectroscopy, between native and denatured states for buried and quenched tryptophan residues as for TIMP-1 [33], and the specificity of binding and inhibitory activity on MMP-2 eventually ensured the correct tridimensional structure. While the tail binds to the physical support, the molecule is free to interact with target molecules. This solution may be very useful for specific sequestering of MMP-2 from any source for analytical or specific pharmacological purposes. The explanation of the restricted specificity of rhTIMP-2 compared with native inhibitor awaits further investigation. But it is evident that, on the one hand, the extra-specially designed NH_2 tail does not impair rhTIMP-2 biological activity towards its elective counterpart. On the other hand, the domain where the tail was added has already revealed the fact that it may undergo modification without loss of biological activity. In fact, the first elucidation of the tridimensional structure of a mutated-deleted TIMP-2 (corresponding to 1–127 amino acids of native protein) showed that the NH_2 domain of DTIMP-2 is exposed to the solvent and does not seem to be essential for molecular interactions [33,34].

In conclusion, the method described here, which makes use of a prokaryotic system and of refolding controlled in the solid phase, has the potential to produce large amounts of active rhTIMP-2. The same procedure may turn out to be useful for a variety of other proteins in which correct disulphide bridging plays a critical role.

Acknowledgements: This research was supported by grants from CNR-ACRO, CNR-Bilaterale Italy–USA, Associazione Italiana per la Ricerca sul Cancro, and MURST, Italy. The authors are grateful to Dr. W.G. Stetler-Stevenson for providing antiserum against TIMP-2, and to G. Walton for revision of the English text.

References

[1] Boone, T., Johnson, M.J., DeClerck, Y.A. and Langley, K.E. (1990) Proc. Natl. Acad. Sci. USA 87, 2800–2804.
[2] Stetler-Stevenson, W.G., Brown, P.D., Onisto, M., Levy, A.T. and Liotta, L.A. (1990) J. Biol. Chem. 265, 13933–13938.
[3] Silbiger, S.M., Jacobsen, V.L., Cupples, R.L. and Koski, R.A. (1994) Gene 141, 293–297.
[4] Curry, V.A., Clark, I.M., Bigg, H. and Cawston, T.E. (1992) Biochem. J. 285, 143–147.
[5] Kleiner Jr., D.R., Unsworth, E.J., Krutzsch, H.C. and Stetler-Stevenson, W.G. (1992) Biochemistry 31, 1665–1672
[6] Albini, A., Melchiori, A., Santi, L., Liotta, L.A., Brown, P.D. and Stetler-Stevenson, W.G. (1991) J. Natl. Cancer Inst. 83, 775–779.
[7] Murphy, A.N., Unsworth, E.J. and Stetler-Stevenson, W.G. (1993) J. Cell. Physiol. 157, 351–358.
[8] Docherty, A.J.P., O'Connel, J., Crabbe, T., Angel, S. and Murphy, G. (1992) Trends Biotechnol. Sci. 10, 200–207.
[9] Docherty, A.J.P., Lyons, J.G., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J.R., Murphy, G. and Reynolds, J.J. (1985) Nature 318, 66–69.
[10] Carmichael, D.F., Sommer, A., Thompson, R.C., Anderson, D.C., Smith, C.G. Welgus, H.G. and Stricklin, G.P. (1986) Proc. Natl. Acad. Sci. USA 83, 2407–2411.
[11] Kohno, T., Carmichel, D.F., Sommer, A. and Thompson, R.C. (1990) Methods Enzymol. 185, 187–195.
[12] Cocuzzi, E.T., Walther, S.E., Rajan, S. and Denhardt, D.T. (1992) FEBS Lett. 3, 375–378.
[13] Kleine, T., Bartsch, S., Bläser, J., Schnierer, S., Triebel, S., Valentin, M., Gote, T. and Tschesche, H. (1993) Biochemistry 32, 14125–14131.
[14] Gomez, D.E., Lindsay, C.K., Cottam, D.W., Nason, M.A. and Thorgeirsson, U.P. (1994) Biochem. Biophys Res. Commun. 203, 237–243.

[15] Cocuzzi, E.T., Walther, S.E. and Denhardt, D.T. (1994) Inflammation 18, 35–43.
[16] Murphy, G., Houbrechts, A., Cockett, M.I., Williamson, R.A., O'Shea, M. and Docherty, A.J.P (1991) Biochemistry 30, 8097–8102.
[17] Willenbrock, F., Crabbe, T., Slocombe, P.M., Sutton, C.W., Docherty, A.J., Cockett, M.I., O'Shea, M., Brocklehurst, K., Phillips, I.R. and Murphy, G. (1993) Biochemistry 32, 4330–4337.
[18] Fridman, R. Bird, RE., Hoyhtya, M., Oelkuct, M., Komarek, D., Liang, C.M., Berman, M.L., Liotta, L.A., Stetler-Stevenson, W.G. and Fuerst, T.R. (1993) Biochem. J. 289, 411–416.
[19] Marston, F.A.O. (1986) Biochem. J. 240, 1–12.
[20] Sambrook, S.D., Fritsch, E.F. and Maniatis, T., (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Stetler-Stevenson, W.G., Brown, P.D., Onisto, M., Levy, A.T. and Liotta, L.A. (1990) J. Biol. Chem. 264, 17374–17378.
[22] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5465–5468.
[23] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff J.W. (1990) Methods Enzymol. 185, 60–89.
[24] Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[25] Sacchi, A., Mauro, F. and Zupi, G. (1984) Clin. Exp. Metastasis 2, 171–178.
[26] Clark, I.M. and Cawston, T.E. (1989) Biochem. J. 263, 201–206.
[27] Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. and Stüber, D. (1988) Bio/Technology 6, 1321–1325.
[28] Burstein, E.A., Vedenkina, N.S. and Ikova, M.N. (1973) Photochem. Photobiol. 18, 263–279.
[29] Brand, L. and Witholt, B. (1967) Methods Enzymol. 11, 776–856.
[30] Howard, E.W. and Banda, M.J. (1991) J. Biol. Chem. 266, 17972–17977.
[31] DeClerck ,Y.A., Yean, T.D., Lee, Y., Tomich, J.M. and Langley, K.E. (1993) Biochem. J. 289, 65–69.
[32] Bodden, M.K., Harber, G.J., Birkedal-Hansen, B., Windsor, L.J., Caterina, N.C.M., Engler, J.A. and Birkedal-Hansen, H. (1994) J. Biol. Chem. 269, 18943–18952.
[33] O'Shea, M., Willenbrock, F.W., Williamson, R.A., Cockett, M.I., Freedman, R.B., Reynolds, J.J., Docherty, A.J.P. and Murphy, G. (1992) Biochemistry 31, 10146–10152.
[34] Williamson, R.A., Martorell, G., Carr, M.D., Murphy, G., Docherty, A.J.P., Freedman, R.B. and Feeny, A. (1994) Biochemistry 33, 11745–11759.