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Role of the 21-kDa protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts

TE-TUAN YANG* AND SUSAN P. HAWKES†

Division of Toxicology, Departments of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446

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ABSTRACT **The 21-kDa protein is an extracellular matrix (ECM) component whose synthesis is stimulated transiently during oncogenic transformation of chicken embryo fibroblasts (CEF) or after treatment of normal cells with the tumor promoter phorbol 12-myristate 13-acetate. Biochemical characterization indicates that the protein is related, but not identical, to two members of the family of tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2. The cDNA of the 21-kDa protein was recently cloned, and based upon its deduced amino acid sequence and other supporting data we propose that it is another member of this family, a TIMP-3. We now report electrophoretic purification of sufficient quantities of this protein to determine its function. The protein promotes the detachment of transforming cells from the ECM. Although its presence in the matrix may be necessary for cell release it is not the only factor involved because it does not influence the adhesive properties of nontransformed cells. It also appears to accelerate the morphological changes associated with cell transformation and stimulates the proliferation of growth-retarded, nontransformed cells maintained under low serum conditions. Based on these data we hypothesize that the 21-kDa protein promotes the development of the transformed phenotype in cultured cells.**

The extracellular matrix (ECM) is a dynamic tissue compartment that functions in the regulation of morphogenesis, differentiation (1), and cell proliferation (2). One of its primary functions is proposed to be the sequestration of growth factors (3) that may be released locally in response to physiological stimuli (4). Cell–ECM interactions also play an important role in diseases involving abnormal growth and development, such as cancer, which is characterized by alterations in the synthesis and degradation of matrix components (5).

We have been studying the potential roles of various ECM components in oncogenic transformation and have reported characterization of the 21-kDa protein found concentrated in the ECM of transforming chicken embryo fibroblasts (CEF) (6, 7). Synthesis of this protein ($M_r \approx 21{,}000$) is stimulated early in the transformation of cells infected with temperature-sensitive mutants of Rous sarcoma virus (RSV) or by treatment of normal, uninfected cells with the tumor promoter phorbol 12-myristate 13-acetate. These observations implicated the 21-kDa protein in the development of the transformed phenotype but did not reveal its precise function.

The NH_2-terminal amino acid sequence of purified 21-kDa protein is >60% identical to a consensus sequence of mammalian tissue inhibitors of metalloproteinases (TIMPs) and the protein displays metalloproteinase inhibitor activity (8). It is the major inhibitor in the ECM and its solubility properties appear unique among inhibitors with a TIMP-like sequence. Its cDNA was recently cloned and sequenced (9), and its deduced amino acid sequence indicates that it is related to, but distinct from, TIMP-1 and TIMP-2. Based on these and other supporting data we propose that the 21-kDa protein is another member of this family, a TIMP-3. To identify it with respect to four other inhibitors that we have detected in this cell system we propose to call it chicken inhibitor of metalloproteinases 3 or ChIMP-3.

The present study addresses the potential function of the 21-kDa protein in the ECM and its role in cell transformation by determining the effect of pure protein on the expression of established transformation parameters by cells in culture. Our data support a role for the protein in the development of the transformed phenotype. Preliminary accounts of some of this work have appeared in abstract form (10, 11).

MATERIALS AND METHODS

Materials. Specific pathogen-free eggs were purchased from Specific Pathogen Free Avian Supplies (Norwich, CT), tissue culture dishes were from Becton Dickinson, medium 199 and sera were from GIBCO Laboratories, and tryptose phosphate broth was from Difco. Phenylmethylsulfonyl fluoride, ε-amino caproic acid, and histone H-1 were from Sigma and electrophoresis supplies and silver stain kits were from Bio-Rad.

Cell Culture. CEF were cultured as described (6, 8). The culture medium, 2-2-1+, is composed of medium 199 supplemented with tryptose phosphate broth (2%), calf serum (2%), chicken serum (1%), and glucose (0.1%). Low serum medium (2-0-0.1+) contains only 0.1% chicken serum. CEF were infected as secondary cultures with the temperature-sensitive mutant of RSV, LA24, clone G2, and experiments were performed with tertiary cells. The 21-kDa protein was purified from cultures seeded at 2×10^6 cells per 100-mm dishes, maintained at 41°C for 12 hr, and transferred to 35°C for 10 hr before preparation of ECM.

Purification of the 21-kDa Protein. ECM was harvested, as described (6, 8), but without trypsin treatment, and solubilized in Laemmli sample buffer (100–200 μl per four dishes) (12), with or without 2-mercaptoethanol. The 21-kDa protein was purified by electrophoresis of ECM samples through a 15% polyacrylamide slab gel (12) and detected by use of a cationic surfactant, as described (8). The bands were excised from the gels and diced into 2-mm cubes, and the protein was eluted in 10 mM sodium acetate buffer, pH 7.4/0.1% SDS at room temperature overnight. The eluate was lyophilized, SDS was removed by addition of cold methanol (90% in H_2O), and the precipitate was stored overnight at −80°C in the methanol. Reduced protein was used for the majority of the isolations as it is more easily located on the gels than nonreduced protein, which migrates as a diffuse band. We are

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Abbreviations: CEF, chicken embryo fibroblasts; ECM, extracellular matrix; EPA, erythroid potentiating activity; MP, metalloproteinase; RSV, Rous sarcoma virus; TIMP, tissue inhibitor of metalloproteinases.

*Present address: Clontech, 4030 Fabian Way, Palo Alto, CA 94303.
†To whom reprint requests should be addressed.

confident that the purified protein refolded correctly as it displayed metalloproteinase inhibitor activity.

Preparation of Substrata. Cells were cultured on four substrata: uncoated plastic dishes and dishes coated with the control protein histone H1, electrophoretically purified 21-kDa protein, or a "mock" sample isolated from a blank gel under identical conditions. Proteins were dissolved in 10 mM sodium phosphate buffer, pH 7.0/9 M urea and sterilized by passage through 0.2-μm membranes (Acrodisc, Gelman). Protein solutions (0.15 ml) were distributed to 60-mm culture dishes and diluted 10-fold by addition of Tris diluent (137 mM NaCl/5 mM KCl/5 mM Na_2HPO_4/25 mM Tris base, pH 7.2) and incubated at 41°C for 4 days. The solution was removed by aspiration and the surface was rinsed with the appropriate medium before seeding cells. Binding of 21-kDa protein to the dishes was monitored by use of ^{35}S-labeled protein; after 12 hr, 2 days, and 4 days, 86.3%, 93.7%, and 94.4% of the protein, respectively, was bound. Amounts of 21-kDa protein determined by the Lowry procedure were significantly higher than those measured by amino acid composition analysis (8). Values from the latter are assumed to be more accurate. Unless specified, the 21-kDa protein was applied to the culture dishes at 0.5 μg per 60-mm dish and histone was applied at 5 μg per dish.

Cell Detachment Assay. Cells (1×10^6 per 60-mm dish in 5 ml of medium 2-2-1+) were incubated at the nonpermissive temperature (41°C) for 6 hr to promote attachment to the culture dishes; then half of the dishes was transferred to the permissive temperature (35°C) for 16 hr to allow development of the transformed phenotype. The assay for cell detachment from the ECM was a modification of published procedures (13, 45). The medium was decanted, the cell monolayer was washed twice with Ca^{2+}-,Mg^{2+}-free phosphate-buffered saline (pH 7.4), and incubated in the same buffer containing 5 mM EGTA (2 ml per 60-mm dish) for 10 min at 41°C. The dishes were agitated on a rotary shaker (Braun Thermonix Shaker 1460) at 120 rpm for 5 min at room temperature. EGTA-released cells were removed and remaining cells were detached by trypsin [0.05% (wt/vol), 41°C, 15 min]. The number of cells in each sample was determined by counting in a Coulter Counter. Cells detached by EGTA treatment are expressed as a percentage of the total cell number.

Cell Proliferation Assay. LA24-infected cells were seeded at 2×10^5 per 60-mm dish in low serum medium (2-0-0.1+), cultured at 35°C or 41°C, detached with trypsin (above), and counted at 24-hr intervals. The population doubling time was determined from a graph of logarithm of cell number versus time in culture. Values are the average of triplicate determinations.

RESULTS

Purification of the 21-kDa Protein. The 21-kDa protein was purified from transforming CEF by preparative gel electrophoresis of ECM components and subsequent elution of the protein from the gel. Analysis of the protein by polyacrylamide gel electrophoresis under reducing and nonreducing conditions indicated a single band with no contaminants detectable by silver staining. Recoveries by this protocol are excellent; >98% of the protein in the preparative gel is recovered in subsequent elution and precipitation steps.

Role of the 21-kDa Protein in Transformation. To examine the role of the 21-kDa protein in cell transformation the properties of LA24-infected CEF seeded on 21-kDa protein-coated substrata were compared with those of cells seeded on control substrata, such as untreated plastic dishes and those treated with histone H1. The latter is similar to the 21-kDa protein in size and pI (the 21-kDa protein has a pI > 9.0; R. M. Malczewski, L. J. Pallanck, and S.P.H., unpublished observations). An additional control of a mock sample isolated from a blank gel was included to ensure that no biologically active components were carried through the purification procedure.

Detachment of Cells from the ECM. During transformation, CEF become progressively less adhesive and can be detached from their substrata by mild agitation with 5 mM EGTA. Under these conditions ≈95% of transforming cells are readily detachable by 30 hr after temperature shift compared to 15% of their nontransformed counterparts (data not shown). To evaluate the potential role of the 21-kDa protein in this process, a time of 16 hr after temperature shift was chosen as a point relatively early in the expression of this transformation parameter. Approximately 15–25% of transforming cells growing on plastic surfaces are readily detachable at this time in comparison to about 1% of their nontransformed counterparts (Fig. 1). The 21-kDa protein further promotes the detachment of transforming cells from 25% to 70%. In four separate experiments this increase ranged from 2- to 5-fold greater than controls without the 21-kDa protein. This effect is not due to a contaminant derived from purification of the protein (gel control) or merely to a small basic protein (histone control) in the matrix. The magnitude of the effect is proportional to the amount of protein bound to the dish; it is linear up to 8–10 pmol of protein per 60-mm culture dish and saturates at ≈15 pmol of protein per dish (Fig. 2). The 21-kDa protein does not enhance detachment of nontransformed cells (Fig. 1).

Morphology. The 21-kDa protein appears to accelerate morphological transformation. When plated on plastic, transforming cells become randomly oriented and refractile by 16 hr after temperature shift (Fig. 3*B*) in comparison to nontransformed cells (Fig. 3*A*). These alterations are more pronounced for transforming cells plated on 21-kDa protein, with the cells beginning to form focal clusters of refractile cells (Fig. 3*D*). Such foci are not usually detected until at least 24 hr in cultures plated on plastic alone (not shown). These observations may reflect altered adhesive properties of the transforming cells, particularly as the 21-kDa protein has no

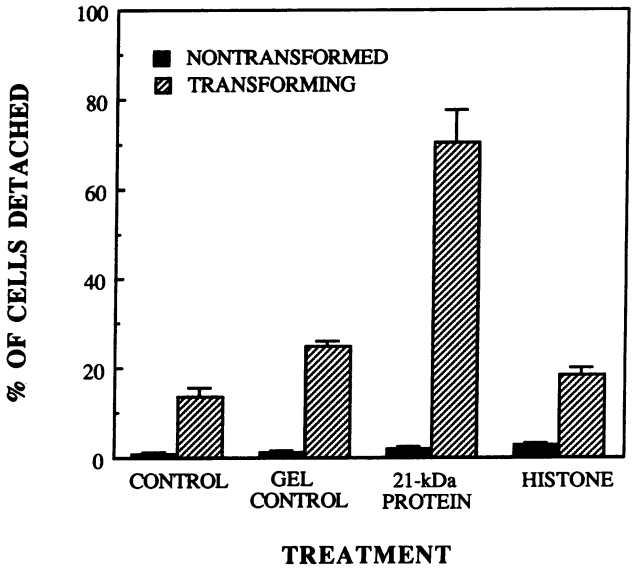

FIG. 1. Effect of the 21-kDa protein on the detachment of cells from the ECM. LA24-infected CEF were incubated on various substrata at 41°C for 6 hr; half of the cultures was transferred to 35°C (transforming) and the other half was maintained at 41°C (nontransformed) for 16 hr. Duplicate cultures were then assayed for cell detachment. Controls included untreated plastic dishes, dishes coated with histone, and dishes treated with a sample eluted from a blank gel.

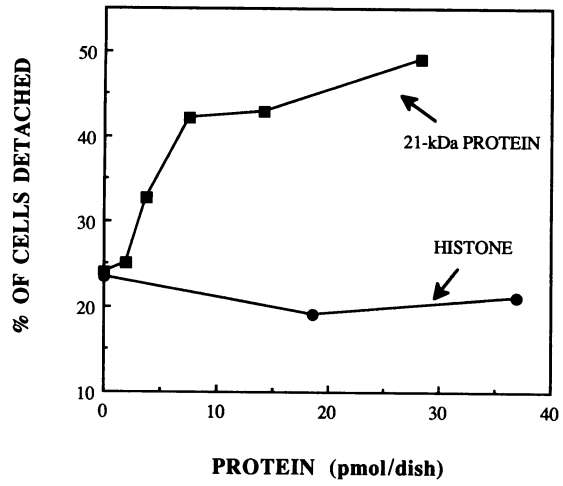

FIG. 2. Concentration dependence of cell–ECM detachment exhibited by transforming cells. LA24-infected CEF were seeded in dishes coated with various concentrations of the 21-kDa protein or the control protein, histone H1. The cells were cultured as indicated in the legend to Fig. 1 and transforming cells (at the permissive temperature of 35°C) were assayed for detachment from the ECM.

detectable influence on the morphology of nontransformed cells (Fig. 3*C*).

Effect of the 21-kDa Protein on Cell Proliferation. Nontransformed LA24-infected CEF (41°C) require serum for proliferation, whereas transforming cells (35°C) do not. Complete medium containing 3.0% serum supports a population doubling time of ≈15 hr for nontransformed cells (data not shown), whereas depleted medium containing 0.1% serum allows cells to divide only every 37–38 hr (Table 1). Under the latter conditions, pure 21-kDa protein stimulates these cells to divide every 15 hr, a rate comparable to that exhibited by transforming cells (Table 1) or nontransformed cells in complete medium. Additionally, the 21-kDa protein stimulates DNA synthesis of nontransformed cells in low serum medium by a factor of at least 2, whereas it progressively inhibits DNA synthesis in transforming cells. The ratios of dpm of [^{3}H]thymidine incorporated into DNA of cells seeded on 21-kDa protein versus plastic substrata at 15 hr, 21 hr, and 28 hr after temperature shift were 2.31, 2.61, and 2.32 for nontransformed cells and 0.94, 0.82, and 0.71 for transforming cells, respectively. Histone also increases the growth rate of the nontransformed cells slightly but its effect is small. The population doubling time of transforming cells is not influenced by added 21-kDa protein. Stimulation of nontransformed cell division is not due to an increased plating efficiency as the 21-kDa protein actually inhibits attachment to culture dishes by ≈15% (data not shown).

Does One Protein Display Metalloproteinase Inhibitor Activity and Promote Cell–ECM Detachment? The method of purification of the 21-kDa protein used in this study differed from that reported earlier for generating the amino acid sequence and composition data and metalloproteinase inhibitory activity (8), primarily in the elimination of trypsin treatment of the ECM. Pure 21-kDa protein, isolated by both procedures, displayed metalloproteinase inhibitor activity and promoted cell detachment. In the latter assay, the sample isolated by the trypsin protocol displayed 80% of the activity of protein purified without this protease, probably as a result of a lower yield or proteolytic inactivation of the protein.

DISCUSSION

The 21-kDa protein promotes the development of the transformed phenotype in CEF infected with a temperature-sensitive mutant of RSV. The protein enhances various parameters such as loss of cell–ECM adhesion and alterations in morphology in transforming cell cultures. The lack of effect on nontransforming cultures indicates that the protein, though perhaps necessary for expression of these properties, is not the only factor involved. Transforming cells must produce another factor(s) that acts with the 21-kDa protein to effect the release of cells from the matrix and to induce morphological changes. The identity of this factor(s) is unknown but it may be related to an association of the 21-kDa protein with hyaluronic acid (unpublished observations). The protein also stimulates proliferation of growth-retarded cells. Nontransformed cells, maintained in low serum medium, divide at a rate comparable to that of transforming cells when the 21-kDa protein is present in the matrix. Additional transformation-associated factor(s) do not appear to be required for this effect. Exogenous 21-kDa protein stimulates proliferation of transforming cells to only a very small extent, presumably because they are producing their own protein and are already growing at near-maximal rate. These data suggest that the protein can replace serum components that are necessary for proliferation of nontransformed cells and that endogenous protein may contribute to the ability of transforming cells to proliferate under low serum conditions.

The 21-kDa protein is a member of the TIMP family of proteins (8, 9). These inhibitors of metalloproteinases are found in body fluids and culture media of tissues and cells from many species (14, 15) and are proposed to play an important role in the regulation of ECM turnover and remodeling. To date, two members have been described, TIMP (otherwise called TIMP-1) and TIMP-2. We propose that the 21-kDa protein is a TIMP-3. Its properties, growth stimulation and promotion of cell transformation, would be expected to involve local ECM remodeling and protease activity, whereas inhibitors of metalloproteinases would be expected to help prevent such activities. Such apparently disparate activities are also expressed by other TIMPs.

TIMP-1 and TIMP-2 also promote cell proliferation in some cell systems. TIMP-1 is identical in sequence to erythroid potentiating activity (EPA) (16), which stimulates growth of erythroid cells *in vitro* and *in vivo* (17–19). TIMP-2 also displays EPA that can be blocked by anti-TIMP-1/EPA antibodies (20). Furthermore, recombinant TIMP-1 stimulates growth of keratinocytes (21) and binds to these cells in a saturable fashion (22). To our knowledge, the effect of TIMP-1 and TIMP-2 on the development of cell transformation in culture has not been reported, although analysis of human tumor samples implicates TIMP-1 in tumorigenesis. Increased expression of TIMP-1 mRNA (23, 24) and protein (25) were observed in human malignant tumors when compared to normal tissues and correlated with clinical aggressiveness (26), although the significance of these data cannot be determined until local concentrations of metalloproteinases are also known. On the other hand, there is considerable evidence to support a role for TIMP-1 and TIMP-2 in suppression of oncogenicity and the invasive phenotype (for review, see ref. 27).

Thus TIMP-1, TIMP-2, and the 21-kDa protein (TIMP-3) display metalloproteinase inhibitory and growth-promoting activities and TIMP-1 and the 21-kDa protein are implicated in tumorigenesis and cell transformation, respectively. To our knowledge, the 21-kDa protein is the only member of this family specifically reported to promote cell–matrix detachment.

The growth-stimulating activity of TIMP-1/EPA (and probably TIMP-2) is thought to be a direct effect on cells via a cell surface receptor and not through metalloproteinase inhibitory activity. It is possible that the 21-kDa protein also stimulates proliferation through a cell surface receptor, either directly or by stimulating production of growth factors. Alternatively, as it is a matrix-specific inhibitor, its mecha-

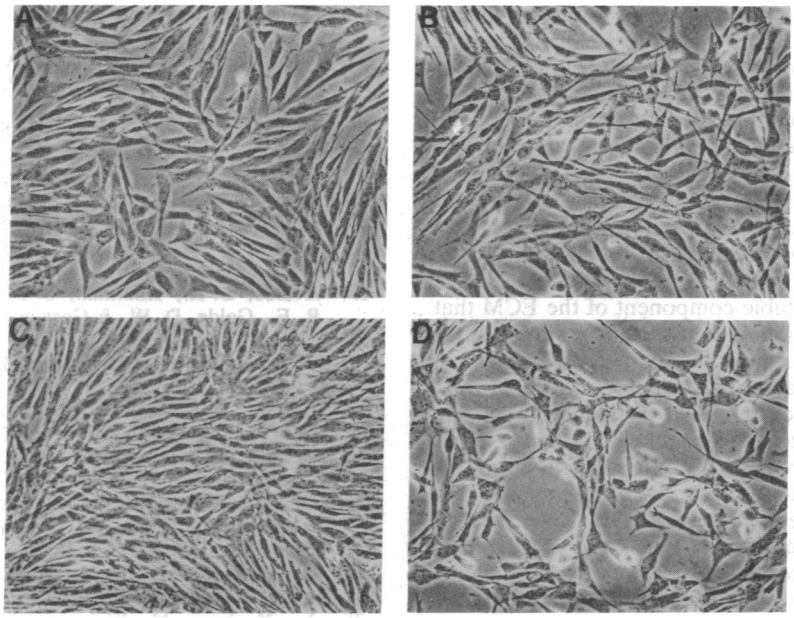

FIG. 3. Morphology of cells cultured on the 21-kDa protein. LA24-infected CEF were seeded in untreated plastic dishes (*A* and *B*) and 21-kDa protein-coated dishes (*C* and *D*). (*B* and *D*) Transforming cells 16 hr after transfer to the permissive temperature, 35°C. (*A* and *C*) Nontransformed cells maintained at the nonpermissive temperature, 41°C. (×350.)

nism of action could be maintenance of ECM integrity, which is essential for supporting cell growth by binding autocrine and/or serum-derived growth factors. There is precedence for protease inhibitors to display growth-promoting activity (28, 29). Experiments to distinguish between these and other possible mechanisms await acquisition of sufficient quantities of the pure native or recombinant protein.

The mechanism by which the 21-kDa protein promotes cell-matrix detachment during cell transformation is also unknown. Preliminary data indicate that the protein binds hyaluronic acid (R. M. Malczewski, L. J. Pallanck, and S.P.H., unpublished observations) and addition of exogenous hyaluronic acid to cultured cells promotes their detachment from the growth substratum (30). Such a mechanism need not necessarily involve its metalloproteinase inhibitory activity. If such activity is essential for cell detachment the latter may be accomplished, as proposed in Fig. 4. The 21-kDa protein may be deposited in the matrix before, or simultaneously with, the release of secreted MP. Alternatively, its interaction may be with membrane-bound enzyme, which would be strategically placed to effect release of cells from the matrix. Such an activity has been reported for RSV-transformed chicken cells (31).

In support of this model, TIMP and MPs are often produced coordinately in response to a single stimulus, such as phorbol 12-myristate 13-acetate (32). Furthermore, RSV-transformed cells display several proteolytic activities including cell surface proteases that promote invasion into ECM (33) and localized degradation of fibronectin at cell–substratum contact sites (34). This adhesive glycoprotein is an obvious target and consistent with this is the correlation between fibronectin-degrading proteases and cell invasion (35). The model is also supported by localization of collagenase at the basal plasma membrane of carcinoma cells (36), the requirement for direct contact of tumor cells with basement membrane for its degradation (37), and the demonstration that surface proteinases beneath tumor cells appear to degrade ECM (38–40).

It has been widely accepted that TIMP-1 inhibits matrix degradation by forming irreversible complexes with MP. However, these complexes are unstable to gel filtration or ion-exchange chromatography (41, 42), particularly if collagen is present. Endothelial cell-stimulating angiogenesis fac-

Table 1. Effect of the 21-kDa protein on growth

Cell phenotype	Substratum	Population doubling time, hr
Nontransformed	Plastic	37.5 ± 2.5
Nontransformed	Histone	31.2 ± 0.0
Nontransformed	21-kDa protein	15.0 ± 3.0
Transformed	Plastic	15.5 ± 1.5
Transformed	Histone	15.0 ± 0.0
Transformed	21-kDa protein	14.0 ± 0.0

LA24-infected CEF were cultured in untreated dishes and dishes coated with 21-kDa protein or histone at the nonpermissive temperature (41°C, nontransformed) or permissive temperature (35°C, transformed) for transformation. At 24-hr intervals the total cell number was counted. Values are expressed as mean ± SEM.

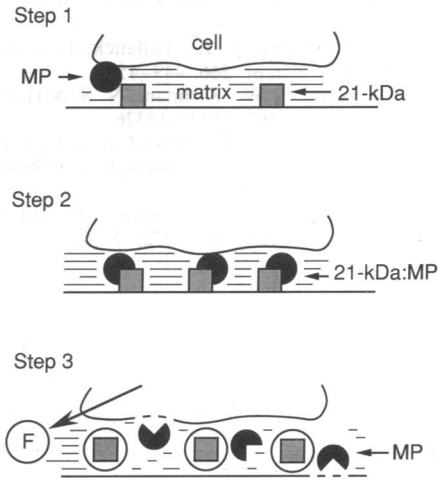

FIG. 4. Model for cell–ECM detachment. The 21-kDa protein is specifically located in the ECM (step 1) and may sequester a metalloproteinase (MP), in an inactive form, beneath cells (step 2). Subsequent release of enzyme from this complex, by a second factor (F), would cause local degradation of proteins involved in cell–matrix adhesion (step 3).

tor, a nonpeptide factor, has been reported to release active MP from its complex with TIMP (43). The small size of this factor ($M_r \approx 400$) would allow it ready access to close contacts between matrix and cell surfaces. A molecule such as this would be a potential candidate for the secondary factor involved in this model of cell detachment during transformation.

One characteristic of the 21-kDa protein that may be crucial for its physiological function is its exclusive location in the ECM. Unlike most other TIMP-related proteins, isolated from tissue fluids or cell culture media, the 21-kDa protein is a relatively insoluble component of the ECM that is not detectable in conditioned media (8). Does the 21-kDa protein promote the transformed phenotype only because of its strategic location? One argument against a role for TIMP-1 in growth control is that it circulates in such large amounts in the body that it is unlikely to be a physiological effector in this process. However, cell receptors for its activity may be expressed only in specific tissues and/or compartments, such as the basal surfaces of cells in contact with ECM. Perhaps other members of this family would promote cell transformation if they were located in the matrix. This concept is supported by the demonstration that J1/tenascin is a multifunctional molecule whose properties depend upon the form of its presentation to the cell, either substrate-bound or in soluble form (44). Whether or not the 21-kDa protein and its TIMP relatives function in a similar manner remains to be determined.

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