The Antitumor Properties of the α3(IV)-(185–203) Peptide from the NC1 Domain of Type IV Collagen (Tumstatin) Are Conformation-dependent

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Tumor progression may be controlled by various fragments derived from noncollagenous 1 (NC1) C-terminal domains of type IV collagen. We demonstrated previously that a peptide sequence from the NC1 domain of the α3(IV) collagen chain inhibits the in vitro expression of matrix metalloproteinases in human melanoma cells through RGD-independent binding to α5β3 integrin. In the present paper, we demonstrate that in a mouse melanoma model, the NC1 α3(IV)-(185–203) peptide inhibits in vivo tumor growth in a conformation-dependent manner. The decrease of tumor growth is the result of an inhibition of cell proliferation and a decrease of cell invasive properties by down-regulation of proteolytic cascades, mainly matrix metalloproteinases and the plasminogen activation system. A shorter peptide comprising the seven N-terminal residues 185–191 (CNYYSNS) keeps the biological activity. As well, the homologous MNYYSNS heptapeptide keeps the β-turn and the inhibitory activity. In contrast, the DNYYSNS heptapeptide, which does not form the β-turn at the YSNS level, is devoid of inhibitory activity. Structural studies indicate a strong structure-function relationship of the peptides and point to the YSNS turn as necessary for biological activity. These peptides could act as potent and specific antitumor antagonists of α5β3 integrin in melanoma progression.

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Tumor growth and metastasis require cross-talk between tumor cells and host stromal cells as well as interactions between tumor cells and the environmental extracellular matrix (ECM), especially basement membranes (1). Basement membranes are thin layers of a specialized ECM which not only provide a supporting structure for various cell types but also largely influence their physiologic behavior. Basement membrane components can also regulate neovascularization, an important process of tumor growth (2, 3). During tumor invasion, the reinforced interactions of tumor cells with basement membranes lead to a sequential proteolytic degradation of their components (4).

The degradation of basement membrane macromolecules involves various proteolytic cascades, such as matrix metalloproteinases (MMPs) and the plasminogen activation system. MMPs are a large family of at least 25 members of zinc-containing endopeptidases that degrade ECM macromolecules. Among them, gelatinase A (MMP-2), gelatinase B (MMP-9), and stromelysin (MMP-3) have widely been shown to play a crucial role in the degradation of basement membranes during melanoma progression (5–7). MMPs are secreted as proenzymes. Their activation, a major step of tumor invasion, results from an imbalance between levels of tissue inhibitors of metalloproteinases (TIMPs) and the MMP activators, such as urokinase, plasmin, or membrane-type MMPs (MT-MMPs). MT-MMPs constitute a new subgroup of MMPs containing an additional domain for anchorage to the plasma membrane. They have been shown to participate actively in basement membrane degradation by directly degrading ECM macromolecules or by activating latent pro-MMP-2 (8–10). On the other hand, the plasminogen activation system is also involved in basement membrane degradation either by direct breakdown of ECM macromolecules or by activating pro-MMPs (11, 12). This system is composed of two different plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), both controlled by plasminogen activator inhibitors (PAI-1 and PAI-2).
Basement membranes are essentially composed of type IV collagen, laminins, entactin/nidogen, and heparan sulfate proteoglycans. Other collagens, such as types XV and XVIII, are localized in the basement membrane area, but their role in the architecture of basement membranes remains to be clearly defined (2). Type IV collagen, a major component of basement membrane, is organized as a network of heterotrimers composed of three α(IV) chains. Each chain possesses a 7 S domain at its N terminus, a large central triple helical domain of about 1,400 amino acids, and a C-terminal noncollagenous (NC1) domain of about 240 amino acids. The heterotrimer is composed of three of six possible α(IV) chains; α1(IV) to α6(IV), each encoded by a specific gene (13). The most widely distributed form of type IV collagen contains two α1(IV) and one α2(IV) chain, whereas other heterotrimers containing α3(IV), α4(IV), α5(IV), and α6(IV) have been described, in glomerular basement membrane, for example (14, 15). The NC1 domain of the α(IV) collagen chains contributes to network polymerization. It is now recognized that NC1 domains from type IV collagen, and also from different collagen types, display several biological activities, mainly by regulating tumor invasion and angiogenesis (16, 17). For instance, NC1 domains from type IV (restin) or type XVIII (endostatin) have been found to inhibit angiogenesis and tumor growth (17). In addition, NC1 domains from α1(IV), α2(IV), or α6(IV) chain of type IV collagen also display antiangiogenic properties, whereas the recombinant NC1 domain of the α3(IV) chain exhibits antiangiogenic and antitumoral properties through two distinct peptide sequences (18–21).

In previous studies, we demonstrated that a specific sequence from the NC1 domain of the α3(IV) chain, comprising residues 185–203, inhibited melanoma cell proliferation as well as melanoma cell invasive properties (22, 23). This inhibitory effect was mediated through binding of the NC1 α3(IV)-(185–203) peptide to the αvβ3 integrin and affected the binding and the activation of pro-MMP-2 at the plasma membrane by decreasing both MT1-MMP and β3 integrin expression (23, 24).

In the present report, we demonstrate that the NC1 α3(IV)-(185–203) peptide inhibits in vivo tumor growth in a mouse melanoma model. The proliferation of B16F1 melanoma cells as well as their migration through Matrigel-coated membranes were significantly decreased. The proteolytic cascade of pro-MMP-2 and the plasminogen activation system (uPA and tPA) were also decreased, both at transcriptional and post-translational levels. A shorter peptide corresponding to residues 185–191 (CNYYSNS) shared the same inhibitory properties. The inhibitory effects were conformation-dependent because the three-dimensional structure of the peptide, mainly at the SNS (189–191) triplet level, which forms a β-turn, was crucial for biological activity. The homologous peptide DNYYSNS, which did not form the β-turn at the SNS triplet, was devoid of inhibitory activity. On the other hand, a MNYSNS peptide, which kept the β-turn conformation, possessed the same inhibitory activity as CNYYSNS.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Culture reagents and molecular biology products were from Invitrogen. Bovine serum albumin (BSA), gelatin and Matrigel (ECM gel) were purchased from Sigma. Human plasminogen was from Calbiochem. Anti-mouse PAI-1 antibody was from American Diagnostica (Andresy, France).

**Peptides**

The NC1 α3(IV)-(185–203) peptide, CNYYSNSYSWLASLNPER, and the corresponding scrambled peptide, YAPLWRNSFPNFLSNVYC, were synthesized by Genospe/É.S.G.S. (Ivry, France). The NC1 α3(IV)-(185–191) peptides CNYYSNS, MNYSNS, and DNYYSNS were obtained by solid phase synthesis using a Fmoc (N-(9-fluorenylmethoxy carbonyl) derivative procedure and were purified further by reverse phase high performance liquid chromatography with a C18 column by elution with a gradient of acetonitrile in trifluoroacetic acid, then lyophilized (25).

The different peptides were modeled with charged amino acids at the extremities (NH$_2$ and COO$^{-}$), according to the chemical synthesis protocol.

**Animals**

Female C57BL6 mice (average body weight, 18–20 g) were purchased from Harlan France (Gannat, France). Animals were individually caged and given food and water ad libitum. They were kept in a room with constant temperature and humidity. All mice were acclimated to our laboratory conditions for 1 week before starting the experiments. The in vivo experiments were conducted according to the recommendations of the Centre National de la Recherche Scientifique.

**Cell Cultures**

B16F1 cells, a lung metastatic subline of murine B16 melanoma, were a generous gift from Dr. M. Grégoire (INSERM UMR 419, Nantes, France). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum in 25-cm$^2$ flasks (Nuncen, VWR International, Strasbourg, France) under a humidified atmosphere of 5% CO$_2$ in air.

Cell proliferation was determined by counting cells with a modified Neubauer cell.

At subconfluence, cells were washed twice with phosphate-buffered saline to remove residual fetal bovine serum and incubated for 48 h in RPMI medium containing 0.5% fetal bovine serum, with or without the NC1 α3(IV) peptides (25 µg/ml). Conditioned media were harvested and centrifuged at 500 × g for 10 min at 4 °C to remove cellular debris. They were then concentrated using Nanosep$^	ext{TM}$ centrifugal devices (VWR International, Mundelein, IL).

| Protein gene studied | Upstream and downstream primers used for semiquantitative RT-PCR analyses | Amplification cycle no. |
|----------------------|--------------------------------------------------------------------------|------------------------|
| β-Actin              | -5'–GTCTGCTGCTTCTCCTTGAGC–3'                                             | 26                     |
|                       | 5'–GACCCATCTCCCTGCTTATGAG–3'                                             |                        |
| uPA                  | -5'–TGGCCAGGAAAATTACCCAGG–3'                                             | 36                     |
|                       | 5'–GCGAATCTGGCCATAGACAC–3'                                               |                        |
| tPA                  | -5'–CCACAGCCTCGTATTCTC–3'                                                | 36                     |
|                       | 5'–TCAGCGCCCTGACAGAAAT–3'                                                |                        |
| PAI-1                | -5'–CTAGAGGACACAGTCTACACTGAG–3'                                          | 36                     |
|                       | 5'–CCACAGCCTCGTATTCTC–3'                                                |                        |
| MMP-2                | -5'–GAAGTTGGGACGTAATACCTG–3'                                             | 42                     |
|                       | 5'–GCCATCCTTCGTAAGACATG–3'                                               |                        |
| MMP-14 (MT1-MMP)     | -5'–GTCCTCTAAGCTCATCCG–3'                                               | 32                     |
|                       | 5'–TTGGATATCCGGCTACATC–3'                                               |                        |
| TIMP-2               | -5'–AGACGTAGTGATACAGGCA–3'                                               | 32                     |
|                       | 5'–GTTCCAGGCGCGAGAAGCAT–3'                                               |                        |

**TABLE I**

**Upstream and downstream primers used for semiquantitative RT-PCR analyses**

| Protein gene studied  | Base sequence of primer | Amplification cycle no. |
|-----------------------|-------------------------|------------------------|
| β-Actin               | -5'–GTCTGCTGCTTCTCCTTGAGC–3' | 26                     |
|                       | 5'–GACCCATCTCCCTGCTTATGAG–3' |                        |
| uPA                  | -5'–TGGCCAGGAAAATTACCCAGG–3' | 36                     |
|                       | 5'–GCGAATCTGGCCATAGACAC–3' |                        |
| tPA                  | -5'–CCACAGCCTCGTATTCTC–3' | 36                     |
|                       | 5'–TCAGCGCCCTGACAGAAAT–3' |                        |
| PAI-1                | -5'–CTAGAGGACACAGTCTACACTGAG–3' | 36                     |
|                       | 5'–CCACAGCCTCGTATTCTC–3' |                        |
| MMP-2                | -5'–GAAGTTGGGACGTAATACCTG–3' | 42                     |
|                       | 5'–GCCATCCTTCGTAAGACATG–3' |                        |
| MMP-14 (MT1-MMP)     | -5'–GTCCTCTAAGCTCATCCG–3' | 32                     |
|                       | 5'–TTGGATATCCGGCTACATC–3' |                        |
| TIMP-2               | -5'–AGACGTAGTGATACAGGCA–3' | 32                     |
|                       | 5'–GTTCCAGGCGCGAGAAGCAT–3' |                        |
**In Vivo Tumor Growth Measurement**

A suspension of B16F1 cells (2.5 × 10⁶ cells in 0.1 ml of RPMI 1640 medium), pretreated or not with the NC1 α₃(IV) peptides (25 μg/ml), was subcutaneously injected into the left side of syngeneic C57BL6 mice. Each group contained at least seven mice. The NC1 α₃(IV) peptides (50 μg in 0.1 ml of RPMI 1640) were injected around the tumor at days 7 and 14. The experiments were repeated twice. Mice were sacrificed at day 21, and tumor sizes were measured. Tumor volume was determined according to Equation 1.

\[ v = \frac{1}{2} L \times W^2 \]  

(Eq. 1)

where \( v \) denotes the volume of the tumor, \( L \) the largest dimension of the tumor, and \( W \) the smallest dimension (27).

**In Vitro Invasion Assays**

Invasion was assayed in modified Boyden chambers (tissue culture-treated, 6.5-mm diameter, 8-μm pore, Transwell, Dutscher, Brumath, France). Briefly, RPMI supplemented with 10% fetal bovine serum and 2% BSA was used as a chemoattractant. 5 × 10⁴ cells were suspended in serum-free RPMI containing 0.2% BSA and seeded onto membranes coated with ECM gel (30 μg/cm²). After a 40-h incubation period, cells were fixed with methanol and stained with crystal violet for 15 min. Cells remaining on the upper face of the membranes were scraped, and those on the lower face were counted using an inverted microscope.

**Zymography Analyses**

**Gelatin Zymography**—The expression of MMP-2 in conditioned media of B16F1 cells, treated or not with the NC1 α₃(IV) peptides (25 μg/ml), was analyzed in gelatin zymography as described previously (23).

The secretion of TIMP-2 was analyzed on B16F1-conditioned media in reverse gelatin zymography as described previously (23).

**Gelatin/Plasminogen Zymography**—For the determination of plasminogen activators, B16F1-conditioned media were analyzed on SDS-polyacrylamide gels containing 1 mg/ml gelatin and 10 μg/ml plasminogen. After electrophoresis, gels were incubated overnight at room temperature in 100 mM glycine buffer, 5 mM EDTA, pH 8.0. Gelatinolytic activity resulting from plasminogen activation was evidenced by white lysis zones after gel staining with G-250 Coomassie Brilliant Blue (28).

**Western Blot Analysis**

Aliquots of concentrated conditioned media were electrophoresed in a 0.1% SDS, 10% polyacrylamide gel. They were then transferred onto
Immobilon-P membranes (Millipore). The membranes were blocked with 5% nonfat dry milk, 0.1% Tween 20 in a 50 mM Tris-HCl buffer, 150 mM NaCl, pH 7.5 (TBS) for 2 h at room temperature, incubated overnight at 4°C with PAI-1 antibody overnight and then for 1 h at room temperature with a second peroxidase-conjugated anti-IgG antibody. Immune complexes were visualized with an ECL chemiluminescence detection kit (Amersham Biosciences).

**RT-PCR Analyses**

Total cellular RNA was extracted with TriReagent (Euromedex, Souffelweyersheim, France) following the manufacturer’s instructions. Total RNA content was evaluated by $A_{260}$ nm measurement and RNA integrity checked by 1% agarose gel electrophoresis.

cDNA was prepared from 1/9262 g of total RNA by reverse transcription (RT) at 42°C for 45 min as described previously (23). RT reaction products (2.5/9262 l) were amplified in a 25/9262 l of PCR mixture in 20 mM Tris-HCl buffer containing 200 μM dNTPs, 0.5 unit of Taq DNA polymerase, and 0.2 μM specific upstream and downstream primers. The downstream primers used are shown in Table I. The PCR was performed in an Eppendorf thermocycler. For each target gene, the kinetics of amplification were performed and the number of cycles selected within the exponential amplification phase (Table I). The amplified DNA fragments were visualized by agarose gel electrophoresis in the presence of ethidium bromide and quantified using Bio-Capt and Bio-1D software (Vilber Lourmat, Marne la Vallée, France).

**Statistical Analyses**

For in vitro experiments, statistical analyses were performed by the Student's t test. Results were expressed as mean ± 1 S.D. For in vivo experiments, volumes of primary tumors were statistically analyzed using the nonparametric $U$ test of Mann and Wittney and the parametric Student’s $t$ test paired with weight-matched mice.
TABLE II
Location of secondary structure elements through the sequence of the NC1 α3(IV)-(185–203) peptide

| Peptide sequence | LINK2  | C | N | Y | S | N | S | Y | S | F | W | L | A | S | L | N | P | E | R |
|-----------------|--------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LINK2           |        |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| MC1             | /      | / | / | * | * | * | / | / | / | / | / | / | / | / | / | / | / | / | / |
| MC2             |        |   |   | / | / | / | * | * | / | / | / | / | / | / | / | / | / | / | / |
| MC3             |        |   |   |   |   | * | * | * | * | / | / | / | / | / | / | / | / | / | / |
| MC4             |        |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| α1             | a1Y  | A | N | A |
| α2             | a2Y  | A | N | K |
| Crystal structure | / | / | * | * | * | * | * | / | / | / | / | / | / | / | / | / | / | / | / |

Circular Dichroism and Secondary Structure Predictions of the NC1 α3(IV)-(185–203) Peptide

The π-π* specific transition of the amide bond of peptides and proteins is sensitive to the local secondary structure of residues. The circular dichroism spectrum of the NC1 α3(IV)-(185–203) peptide was recorded on a JASCO 810 dichrograph spectrometer at room temperature using a cylindric cell of 0.1-cm path length. The sample was prepared at a concentration of 0.1 mM in a 25 mM acetic acid aqueous solution to reach a final pH 5.0. Data presented are the results of nine successive time accumulations in the 185–260 nm spectral range and are expressed as ε, the molar ellipticity (in deg cm^2 dmol^-1). Secondary structure percentage contents were deduced from the CD data using the CONTIN program (29). Adjusted local secondary structure predictions were performed using the LINK2 method (30), and β-turn predictions were performed by the COUDES propensity method. This software predicts the β-turn occurrence in peptides and proteins by successively performing secondary structure predictions through the sequence using the GOR III method (31) and then localizing the potential turns (locations and types) in the unordered (neither helical nor strand) regions.

Monte Carlo Simulations of the NC1 α3(IV)-(185–203) Peptide

Monte Carlo simulations of the NC1 α3(IV)-(185–203) peptide were performed using the method described by Derreumaux (32), which is efficient for peptides up to 20 amino acids.

Homology Modeling of the NC1 α3(IV)-(185–203) Peptide

The NC1 α3(IV)-(185–203) peptide was modeled by homology, using the crystal structure of the NC1 (α1IV)2 (α2IV)2 collagen hexameric structure from Than et al. (33) (PDB 1LI1) as a template. Considering that each of the NC1 domains of the αIV chains could be divided into two homologous subdomains (34), we performed a multiple sequence alignment of the six (two α1IV, two α2IV, and two α3IV) subdomains. The corresponding structures extracted from the crystal data were then restricted to the region corresponding to the NC1 α3IV (185–203) peptide. After a sequence-based structural fit (see Fig. 7A), all the four reference structures corresponding to the α1IV (blue, magenta) and α2IV (red, yellow) homologous subdomains were found to be closely related with a root mean square deviation (r.m.s.d.) ranging from 0.4 to 1.18 Å. Ca coordinates of the NC1 α3IV (185–203) peptide were then assigned using the HOMOLOGY module of the INSIGHT II software (Accelrys).

Molecular Dynamics Simulations

All peptides were modeled on a Silicon Graphics OCTANE DUO work station using the CHARMM program (35), version c27b3. Molecular dynamics simulations were then performed using the CHARMM parallel (C27b3) software on a Sunfire 6800 computer (SMP 24 x UltraSparc III @ 900 MHz, 24 Go), at physiological temperature (310 K), using the Verlet algorithm and an integration step of 1 fs. All atoms were explicitly included in the calculation with water molecules modeled using the classical TIP3P Jorgensen’s water potential (36).

Starting structures for the NC1 α3IV (185–203) peptide were those deduced from Monte Carlo simulations; lateral chains were reconstructed using the default parameters as found in the CHARMM parameter file (37). The different structures were then minimized, first constraining the backbone atoms to allow the side chains to adapt and second with all constraints removed. Folding of the NC1 α3IV-(185–196) peptide was studied using the homology model of the NC1 α3IV-(185–203) peptide as a starting structure, reduced to its 12 first residues.

In each case, the system was minimized, first with the protein atoms fixed and then with the solvent fixed. A final minimization was performed with all constraints removed. The system was then heated to 310 K in a 15-ps heating phase and equilibrated in two 40-ps stages, namely early and late equilibration, in which the velocities were first assigned using a Gaussian distribution and then scaled every 50 steps to keep the temperature at 310 ± 10 K. A force switching function for electrostatics and a potential shifting function for Van der Waals interactions were used, both applied between 10 and 12 Å. Periodic boundary conditions were applied during all of the simulation stages.

Adiabatic Map Calculation

For each of the NC1 α3IV (185–191) (CNYSNNS, MNYSNNS, and DNYYSNS) peptides, we performed a quasi-exhaustive conformational search. Rather than assuming that each residue can adopt any dihedral angle values in the Ramachandran map, we assumed that each nonterminal residue could adopt one among the only 10 possible (ϕ, χ) couples of values corresponding to the regions of the Ramachandran map which are commonly explored by protein classical residues (32). For each heptapeptide, we got 100,000 possible conformations. All of these obtained conformations were constructed and energy minimized using the CHARMM program (35). Minimization was done in implicit solvent with a dielectric constant ε = 80.0. Backbone dihedral angles were first constrained using a force constant of 5,000 kcal mol^-1, progressively relaxed during several successive steepest descent and adopted basis Newton Raphson minimization cycles. A final minimization stage was performed with all constraints removed. Minimization was stopped when the energy gradient was lower than 10^-4 kcal mol^-1.

For each peptide, the 1,000 best conformations (presenting the lowest energies) were kept for analysis and were reduced into 10 families of representative structures by using the clustering procedure described below. A Boltzmann’s weight was then calculated for each family of structures. First, for each of the 1,000 conformations, we computed

$$\Delta E(n) = E_{min} - E(n)$$  \hspace{1cm} (Eq. 2)

where $E_{min}$ is the energy of the global energy minimum, and $E(n)$ the energy of the n-th conformation. Then, for all the $n = 1,000$ conformations, we computed

$$Z = \sum_{n=1}^{N} e^{-\Delta E(n)/kT}$$  \hspace{1cm} (Eq. 3)

where k is the Boltzmann’s constant and T the temperature. For each of the 10 families of structures, we calculated

$$Z_i = \sum_{n=1}^{N} e^{-\Delta E(n)/kT}$$  \hspace{1cm} (Eq. 4)

where I is the total number of conformations in the family. Finally, the relative Boltzmann probability for each family is given by Equation 5.

$$P_{int} = Z_i/Z$$  \hspace{1cm} (Eq. 5)

Results were expressed as percentages, for convenience.

Clustering Method

The whole sets of structures explored either by molecular dynamics simulations, or by adiabatic map calculation, were grouped into a re-
duced number of representative structures using a clustering procedure. For this purpose, each pair of conformations, named \(i\) and \(j\), were optimally fitted on their heavy atoms positions, and the corresponding r.m.s.d. values were stored into a two-dimensional matrix. A given element on the matrix diagonal represents the r.m.s.d. between a given conformation and itself, that is r.m.s.d. \((i, i)\) and consequently presents a zero value, and an off-diagonal element represents the r.m.s.d. between two different conformations, r.m.s.d. \((i, j)\). Thus, for the set of \(N\) conformations, each line of the resulting \((N \times N)\) matrix contains the dissimilarities of one conformation to all the others. Thus, each line was also taken as a vector of \(N\) descriptors used to construct a phylogenetic tree based on these dissimilarities, which was cut further to obtain the chosen number of 10 representative families of conformations. The structures belonging to each group were kept into separate files for analysis. For each family, we calculated and graphed the corresponding mean structures using the MoelMol program (38).

RESULTS

We demonstrated previously that the NC1 \(\alpha(IV)-(185–203)\) peptide inhibited the in vitro proliferation of various cancer cell lines (22, 39) and the invasive properties of human HT-144 melanoma cells by decreasing both the binding of pro-MMP-2 to the cell membrane and interactions with \(\alpha_\text{IIb} \beta_3\) integrin (23). In this paper, we studied the in vivo effects of various NC1 \(\alpha(IV)\) heptapeptides in a mouse melanoma model and the relationship between the inhibitory activities of the peptides and their structures.

Inhibition of In Vivo Tumor Growth by NC1 \(\alpha(IV)\) Peptides—The effects of NC1 \(\alpha(IV)\) peptides on in vivo tumor growth were studied in a mouse melanoma model. Murine B16F1 melanoma cells, pretreated or not with the different peptides, were subcutaneously injected into the left side of syngeneic C57BL6 mouse, and tumor volumes were measured at day 21. Untreated B16F1 cells induced the development of a subcutaneous tumor. The NC1 \(\alpha(IV)-(185–203)\) peptide inhibited tumor growth by 43\% \((p < 0.01)\) (Fig. 1), whereas the corresponding scrambled peptide had no effect. The shorter heptapeptide NC1 \(\alpha(IV)-(185–191)\) CNYSYSNS also induced a 64\% inhibition of tumor growth \((p < 0.01)\). The substitution C185M did not modify the amplitude of the inhibition, whereas the substitution C185D abolished the inhibitory effect.

Inhibition of in Vitro Proliferation of B16F1 Melanoma Cells by NC1 \(\alpha(IV)\) Peptides—The in vitro proliferation of B16F1 cells was significantly inhibited by the NC1 \(\alpha(IV)-(185–203)\) peptide \((-28.5\%, p < 0.01)\), whereas the scrambled peptide had no significant effect (Fig. 2). The shorter heptapeptide CNYSYSNS, as well as its analogous peptide MNYYSNS, also inhibited cell proliferation \((-22.4\%\) and \(-27\%,\) respectively, \(p < 0.01)\). In contrast, the heptapeptide DNYYSNS had no significant effect.

Inhibition of in Vitro Migration of B16F1 Melanoma Cells by NC1 \(\alpha(IV)\) Peptides—The effect of NC1 \(\alpha(IV)\) peptides on B16F1 cell migration through an in vitro reconstituted basement membrane was analyzed on modified Boyden’s chambers, using Matrigel-coated membranes and a gradient of 2\% BSA and 10\% fetal calf serum as a chemoattractant. Migration was assessed after 40 h. The NC1 \(\alpha(IV)-(185–203)\) peptide led to a large decrease \((-38\%; p < 0.01)\) in B16F1 cell migration, as well as the CNYSYSNS or MNYYSNS peptides \((-48.2\%\) and \(-39.8\%,\) respectively, \(p < 0.01)\). The peptide DNYYSNS was devoid of inhibitory effect (Fig. 3).

Effect of NC1 \(\alpha(IV)\) Peptides on Protease Expression—Gelatin zymography analysis of MMPs in the conditioned media of B16F1 cells (Fig. 4A) showed that the secretion of pro-MMP-2 was decreased by NC1 \(\alpha(IV)-(185–203)\), CNYSYSNS, and MNYYSNS, but not by DNYYSNS. The secretion of uPA and tPA was inhibited by the same peptides as pro-MMP-2 (Fig. 4B). By reverse gelatin zymography, we also found that the secretion of TIMP-2 was not altered, as well as the secretion of PAI-1 analyzed by Western blot (data not shown).

Semiquantitative RT-PCR analysis confirmed the decreased expression of pro-MMP-2, uPA, and tPA in B16F1 cells incubated with NC1 \(\alpha(IV)-(185–203)\), CNYSYSNS, and MNYYSNS, but not with DNYYSNS (Fig. 4C). No alterations of TIMP-2 or PAI-1 were observed (data not shown).

Structural Study of the NC1 \(\alpha(IV)-(185–203)\) Peptide—The CD spectrum of the NC1 \(\alpha(IV)-(185–203)\) peptide (Fig. 5) was characterized by an intense positive signal below 195 nm and by two negative bands around 200 and 220 nm; these results indicated a mixture of \(\alpha\), \(\beta\), and coil structures with the presence of some turns. The CONTIN program (29) gave 10, 40, and 50\% of \(\alpha\), \(\beta\), and undetermined structures (coil), respectively. The adjusted secondary structure predictions using the LINK2 method (30) led to the localization of two short \(\beta\)-strands on the CN and the YSFWL residues (see Table II). Moreover, these results indicated a propensity to form an \(\alpha\)-helix at the C-terminal end of the peptide (on the E and R residues). Thus, undetermined structures and consequently loop or turn conformations were more precisely predicted in the YSYSNS (187–191) and the ASLNP (197–201) regions of the peptide. The \(\beta\)-turn predictions pointed to the YSNS (type IV \(\beta\)-turn) and the SLNP (type VIII \(\beta\)-turn) tetrapeptides as the most likely turns for the first and the second parts of the peptide, respectively.

Stable conformations of a molecular system, which determine its behavior in solution, localize on minima points of its energy surface. To identify these points, which correspond to the most probable conformations of the NC1 \(\alpha(IV)-(185–203)\) peptide, we used Monte Carlo simulations (32). Four energy-related conformations were identified (Fig. 6), with a 2.3 Å mean r.m.s.d. on their backbone atoms. We used these reference structures as starting points for short molecular dynamics simulations ranging from 700 to 1,000 ps, which also confirmed their stability in water (data not shown). Two models, MC1 and MC2 (for their secondary structures, see Table II) were characterized by an antiparallel \(\beta\)-sheet motif in their 12 first residues, giving rise to a \(\beta\)-turn around the SNS residues. This turn was more precisely localized either on the NSYS (model MC1) or the YSNS residues (model MC2). The two other models (MC3 and MC4) were less structured but nevertheless presented the same ability to form a turn around the SNS region of the chain. Moreover, we noted the presence of some \(\alpha\)-helical conformation in the second part of the chain in three of the four models. Through these observations, we could conclude that our Monte Carlo results were fully consistent with our CD spectrum and the corresponding LINK2 adjusted secondary structure predictions, which presented some \(\alpha\), \(\beta\), and coil.

FIG. 6. Structural fit on the backbone atoms of the lowest energy conformations of the NC1 \(\alpha(IV)-(185–203)\) peptide obtained by Monte Carlo simulations (mean r.m.s.d. = 2.33 Å). \(\beta\)-strands organized into \(\beta\)-sheets are symbolized as blue arrows, \(\alpha\)-helical conformation as a red-yellow helix. These four structures correspond to those in Table II.
secondary structures occurring in the same regions as described above (see Table II). Moreover, considering independently the nature of their secondary structure, our models also agree with the observed α1(IV) and α2(IV) chain secondary structure elements extracted from the x-ray data (33). These observed secondary structure elements are also reported in Table II. The presence of these two strands as well as the presence of a turn on the YSNS homologous residues (YANA for α1 and YANK for α2 chains, respectively) indicated that the free peptide in solution might easily adopt a β-sheet motif in the first part of its chain during its folding process.

Structural Study of the NC1 α3(IV)-(185–196) Peptide—We went on with the folding of the NC1 α3(IV)-(185–203) peptide by molecular dynamics simulation. The starting model was constructed by homology modeling, on the basis of the crystallographic data (Fig. 7A) (33).

Folding of the structure obtained was followed by only considering its 12 first residues (185CNYYSNSYSFWL196) for a convenient computational time. A molecular dynamics simulation of this peptide was then performed in explicit water and lasted for 4 ns. As a main result, we showed that the YSNS turn initially present in the starting structure, drove the folding of the NC1 α3(IV)-(185–196) peptide as indicated by the corresponding representative structures in Fig. 7B. The YSNS turn was observed along nearly all of the trajectory and was stabilized by a H-bond at regular intervals of time.
Comparative Study of NC1 α3(IV)-(185–191) Heptapeptides

CNYYSNS, MNYYSNS, and DNYYSNS—Adiabatic maps of the three modified NC1 α3(IV)-(185–191) peptides resulted in different structures for the CNYYSNS and MNYYSNS peptides on one hand and the DNYYSNS on the other hand. For each of the three peptides, the 1,000 lowest energy conformations from the adiabatic map calculation were reduced to 10 families of representative structures (referred to as F1–F10); their calculated Boltzmann weight values are reported on Fig. 8A.

For each peptide, F1 refers to the group of conformations which included the global energy minimum determined by the method. For both the CNYYSNS and the MNYYSNS peptides, the groups (F1-▲, F1-● respectively) were the most important,
with Boltzmann weights reaching 88 and 98.5%, respectively. For the DNLYSNS peptide, the F1- group was only 19.5%, indicating a less populated minimum energy state. These results also indicated that both the CNYYSNS and MNYYSNS peptides might give rise to a quasi-unique type of structure represented by their global energy conformation, whereas the DNLYSNS peptide was able to form several different stable states, in equilibrium.

To determine which representative groups were found in common among the three peptides, we graphed the corresponding phylogenetic tree, in which each family is represented by its lowest energy conformation (Fig. 8B). We showed that both the CNYYSNS and MNYYSNS peptides shared a closely related minimum energy conformation (F1- and F1-), which also present a YSNS turn. The F1- and F2- most probable structures for the DNLYSNS peptide were far away in the tree, indicating a completely different structure. The most related conformation for DNLYSNS was F7-, but this conformation presented a very low probability (p = 2%), indicating that this structure might not be favored for the DNLYSNS peptide.

DISCUSSION

Tumor progression is a multistep process characterized by interactions of tumor cells with the environmental extracellular matrix or host stromal cells. The interactions involve ECM macromolecule domains released, for example, from basement membrane components, by a limited and directed proteolysis. At present, basement membranes must be considered not only as a supporting network for various cell types, but also as a potential regulator of cell behavior. Among basement membrane components, type IV collagen provides an architectural network, but the different NC1 domains of its constitutive (IV) chains might also regulate cellular functions and exhibit antiangiogenic or antitumor properties (16). We demonstrated previously that the NC1 α3(IV)–(185–203) peptide inhibited in vitro polymorphonuclear neutrophil respiratory burst and granule exocytosis (41). The binding of the NC1 α3(IV)–(185–203) peptide to the receptor complex CD47/αβ3 integrin induced an "outside-in" transduction signal characterized by an increase of intracellular calcium and cyclic AMP concentrations (42). The NC1 α3(IV)–(185–203) peptide also inhibited in vitro proliferation of melanoma cells and various cancer cell lines and their invasive properties (22–24). Using monoclonal antibodies directed against αβ3 integrin subunits or CD47, we could demonstrate that the CD47-αβ3 integrin complex also served as a receptor for the NC1 α3(IV)–(185–203) peptide at the melanoma cell surface (43). Furthermore, the binding of the NC1 α3(IV)–(185–203) peptide occurred on the β3 integrin subunit independently of CD47 and the α subunit associated with β3 (24). It was also independent of the RGD peptide recognition sites, as confirmed by other authors (44).

In the present paper, we demonstrate that the NC1 α3(IV)–(185–203) peptide is also capable of inhibiting in vivo tumor growth in a mouse melanoma model. The inhibitory activity was reproduced by the NC1 α3(IV)–(185–191) heptapeptide, containing the seven N-terminal amino acids CNYYSNS of the NC1 α3(IV)–(185–203) peptide. The inhibition of tumor growth might result from a decrease of pro-MMP-2 secretion and a decrease of the plasminogen activation system (uPA and tPA) in tumor cells. In previous studies, we showed that the suppression of the Cys185 residue of the peptide abolished the inhibitory activity of NC1 α3(IV)–(185–203) (23, 41). On the other hand, we also showed that the SNS (189–191) triplet was crucial for the biological activity of the peptide, because the substitutions S189A or S191A abolished the activity (23, 41).

We also studied the importance of the side chain of the Cys185 residue. For this purpose, we synthesized homologous peptides, in which Cys185 was replaced either by a Met residue to test the role of the presence of the sulfur atom, or by a Asp residue to test the role of cation binding of the lateral chain. The modified MNYYSNS peptide reproduced the inhibitory activity of the wild type CNYYSNS peptide. On the contrary, the substitution of Cys185 by an Asp residue, resulting in a DNLYSNS peptide, totally inhibited the inhibitory activity both in vitro and in vivo. These data suggest an important role of the sulfur atom in the conformation of these peptides. Accordingly, we went on with structural studies of the different peptides that were studied at the biochemical level, to establish a structure-function relationship. Local secondary structures of the NC1 α3(IV)–(185–203) peptide adjusted by CD data on one hand, and Monte Carlo simulations on the other hand, strongly support the hypothesis of a β-turn located on the YSNS residues. Because these residues are also crucial for the biological activity of this peptide, we therefore propose that the specific local secondary structure motif that was observed during all our dynamics trajectories, may be important for the peptide-αβ3 integrin interactions. Results of the quasi-exhaustive conformational search for the three NC1 α3(IV)–(185–191) heptapeptides confirmed the hypothesis by showing that the CNYYSNS and MNYYSNS peptides that possess the biological activity, adopted this particular structure. On the contrary, the ineffective DNLYSNS peptide did not adopt such a structure. These results led us to think that the sulfur atom present in the first residue of the peptide might also be important for the local conformation of the peptide and not for the direct interaction with the receptor.

The structural studies also indicate a structure-function relationship for the NC1 α3(IV)–(185–203) or NC1 α3(IV)–(185–191) CNYYSNS peptides and strongly point to the YSNS turn as responsible for the biological activity. We suppose that the three hydroxyl groups of the YSNS peptide tetrapeptide are crucial for the peptide-αβ3 integrin interaction, by directly interacting with residues on the surface of the β3 subunit. In fact, the β-turn located in this region of the α3(IV) collagen chain perfectly exposes these residues for such an interaction. Collectively, these results demonstrate a strong structure-function relationship for the NC1 α3(IV)–(185–203) or NC1 α3(IV)–(185–191) CNYYSNS peptides and suggest these peptides as potent and specific antitumor antagonists of the αβ3 integrin in melanoma progression.

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REFERENCES

1. Steiber Stevenson, W. G., Amavoorian, S., and Liotta, L. A. (1991) Ann. Rev. Cell Biol. 9, 541–573
2. Marneros, A. G., and Olsen, B. R. (2001) Matrix 20, 337–345
3. Carmeliet, P., and Jain, R. K. (2000) Nature 407, 249–257
4. Horner, W., Ennor, H., Mommaisse, J. C., and Bellon, G. (2002) Semin. Cancer Biol. 13, 11. 9
5. Hofmann, U., Westphal, J. R., Van Muijen, G. N. P., and Ruiter, D. J. (2000) J. Invest. Dermatol. 115, 537–544
6. Vihinen, M., and Kahari, V. M. (2002) Int. J. Cancer 99, 157–166
7. Egblad, M., and Werb, Z. (2002) Nat. Rev. Cancer 2, 161–174
8. Ohuchi, E., Inai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997) J. Biol. Chem. 272, 2446–2451
9. Deryugina, E. I., Ratnikov, B., Monosov, E., Postnova, T. I., Disepri, R., Smith, J. W., and Strongin, A. Y. (2001) Exp. Cell Res. 263, 209–223
10. Seiki, M. (2002) Curr. Opin. Cell Biol. 14, 624–632
11. De Vries, T. J., Van Muijen, G. N. P., and Ruiter, D. J. (1996) Melanoma Res. 6, 79–88
12. Andreassen, T. A., Kjoller, L., Christensen, L., and Duffy, M. J. (1997) Int. J. Cancer 72, 1–22
13. Hudson, B. G., Reinders, S. T., and Tryggvason, K. (1993) J. Biol. Chem. 268, 26033–26036
14. Boustaud, A., Borza, D. B., Bondar, O., Gunwar, S., Netzer, K. O., Singh, N., Ninomiya, Y., Sado, Y., Noelken, M. E., and Hudson, B. G. (2000) J. Biol. Chem. 275, 30716–30723
15. Borza, D. B., Bondar, O., Todd, P., Sundaramoorthy, M., Sado, Y., Ninomiya, Y., and Hudson, B. G. (2002) J. Biol. Chem. 277, 40075–40083
16. Ortega, N., and Werb, Z. (2002) J. Cell Sci. 115, 4201–4214
