Cyclic and linear peptides containing the Asn-Gly-Arg (NGR) motif have proven useful for delivering various anti-tumor compounds and viral particles to tumor vessels. We have investigated the role of cyclic constraints on the structure and tumor-homing properties of NGR peptides using tumor necrosis factor-α (TNF) derivatives containing disulfide-bridged (CNGRC-TNF) and linear (GNGRG-TNF) NGR domains. Experiments carried out in animal models showed that both GNGRG and CNGRC can target TNF to tumors. However, the anti-tumor activity of CNGRC-TNF was >10-fold higher than that of GNGRG-TNF. Molecular dynamic simulation of cyclic CNGRC showed the presence of a bend geometry involving residues Gly3-Arg4. Molecular dynamic simulation of the same peptide without disulfide constraints showed that the most populated and thermodynamically favored configuration is characterized by the presence of a β-turn involving residues Glyβ-Argβ and hydrogen bonding interactions between the backbone atoms of Asnβ and Cysγ. These results suggest that the NGR motif has a strong propensity to form β-turn in linear peptides and may explain the finding that GNGRG peptide can target TNF to tumors, albeit to a lower extent than CNGRC. The disulfide bridge constraint is critical for stabilizing the bent conformation and for increasing the tumor targeting efficiency.

Phage display peptide libraries are commonly used to obtain peptide sequences interacting with proteins differentially expressed in normal and pathological tissues (1, 2). For instance, in vivo panning of phage libraries in tumor-bearing animals have proven useful for selecting peptides able to interact with proteins expressed within tumor-associated vessels and to home to neoplastic tissues (3). Among the various tumor targeting ligands identified so far, the CNGRC peptide have proven useful for delivering various anti-tumor compounds, like chemotherapeutic drugs, apoptotic peptides and cytokines, to tumor vessels (3–5). For example, we have recently shown that targeted delivery of tumor necrosis factor-α (TNF) to tumor vasculature can be obtained by coupling its N terminus to the C terminus of the CNGRC peptide, by genetic engineering technology (5). This approach markedly improved the therapeutic index of TNF in animal models, either when used alone (5) or in combination with chemotherapeutic agents (6). Studies on the mechanism of action showed that the targeting domain of this TNF derivative (called NGR-TNF) binds an aminopeptidase (CD13) isof orm expressed in tumor vessels, and not other isoforms expressed in normal epithelia or myeloid cells (7). Besides CNGRC, other tumor vasculature targeting peptides containing the NGR motif have been identified, such as linear NGRAHA and cyclic CVLNGRMEC (3). These and other linear and cyclic NGR peptides have been used for targeting viral particles to endothelial cells (8, 9). Although these findings may suggest that peptide cyclization is not necessary for the targeting properties of NGR peptides, the role of cysteines and the disulfide bridge on structure and activity of the NGR motif still remains to be investigated. To examine the effect of cyclic constraints on the targeting properties of NGR peptides, we have compared the anti-tumor activity of cyclic CNGRC-TNF and linear GNGRG-TNF conjugates, prepared by recombinant DNA technology, using a mouse model. In addition, to explore the dynamic behavior and the conformational characteristics of NGR peptides with or without cyclic constraints, we performed molecular dynamic (MD) simulations of two CNGRC peptides, one without covalent bond between Cys1 and Cys5 (called “NoSS”), and one with disulfide-bridged cysteines (called “SS”). We provide evidence to suggest that the NGR sequence has a strong propensity to form β-turns that favor the formation of intramolecular hydrogen bonding interactions. However, although the most populated configuration of both NoSS and SS peptides are characterized by a bend geometry involving residues Glyβ-Argβ, we show that GNGRG is less efficient than CNGRC in targeting TNF to tumor animal models, indicating that the disulfide bridge is critical for the in vivo targeting activity of the NGR motif.

**EXPERIMENTAL PROCEDURES**

**Preparation and Characterization of Synthetic Peptides**—The synthetic peptides used in this study were produced by Primm (Milan, Italy). Peptide concentrations were checked using the Protein Assay kit (Roche Molecular Biochemicals). Mass spectrometry analysis of CNGRC, GNGRG, and RGNG peptides indicated a molecular mass of 550.23 ± 1 Da (expected for disulfide-bridged peptide, 551.18 Da), 460.15 ± 1 Da (expected, 459.2 Da), and 459.54 ± 1 Da (expected, 459.2 Da), respectively. Free sulfhydryl groups in the CNGRC peptide were <0.1%, as checked by titration with Ellman’s reagent (Pierce) (11).

**Immunohistochemical Studies—**Immunohistochemical analysis of renal cell carcinoma was performed as described (7) using the anti-CD13 mAb WM15 (PharMingen, San Diego, CA).
Preparation and Characterization of Human TNF, CNGRC-TNF, and GNGRG-TNF — The cDNA coding for CNGRC-TNF (consisting of human TNF fused with the C terminus of GNGRCG) was prepared by recombinant DNA technology as described (5). The cDNA coding for GNGRG-TNF (human TNF fused with the C terminus of GNGRGG) was obtained by PCR on CNGRC-TNF plasmid, using the following primers: 5′-TATACATATGCGACGCGCGCGGTCG-3′ (5′ primer); 5′-GGACGCGCCGCTGAGATCCCGGTGTAG-3′ (3′ primer). Sequences were designed to include the NdeI and BamHI restriction sites (underlined) for the cloning into a pET11 plasmid, digested with the same enzymes. The NdeI site also contains the translation start codon. TNF and CNGRC-TNF/CDNA were expressed in E. coli cells and purified from cell extracts as described (5). The CNGRC-TNF/CDNA were expressed in E. coli cells and purified from cell extracts by affinity chromatography on soluble p55-TNF receptor (st-TNF-R1)—Sepharose as follows: 5 mg of recombinant st-TNF-R1 was prepared as described (39), and coupled to 2 ml of activated CH-Sepharose (Amersham Biosciences Europe GmbH, Freiburg, Germany) according to the manufacturer’s instructions. The column was washed extensively with sterile and endotoxin-free phosphate-buffered saline, loaded with GNGRG-TNF crude extract, dialyzed in phosphate-buffered saline, and desorbed by elution with 7 M urea, 100 mM Tris-HCl, pH 8. Both CNGRC-TNF and GNGRG-TNF were denatured with 7 M urea and refolded as described (5). Briefly, the denatured products were dialyzed against 2.5 M urea, 100 mM Tris-HCl, pH 8.0, at 4 °C (140 mM NaCl) for 3 days. After dialysis, 1.5 M urea, 100 mM Tris-HCl, pH 8.0 (16 h), and 1 M urea, 100 mM Tris-HCl, pH 8 (140 min). Finally the products were dialyzed against 100 mM Tris-HCl, pH 8.0 (16 h). The products were centrifuged at 13,000 × g (30 min, 4 °C), filtered through a 0.22 μm membrane (Nalgene). All solutions used in the purification and refolding steps were prepared with sterile and endotoxin-free water (S.A.L.F. Laboratorio Farmacologico SpA, Bergamo, Italy). Protein concentration was measured using the BCA protein assay reagent (Pierce). Protein purity and identity were checked by SDS-PAGE, Western blotting, and electrospray mass spectrometry (ESI-MS). The in vitro cytolytic activity of TNF, CNGRC-TNF, and GNGRG-TNF were measured by standard cytolytic assay with L-M mouse fibroblasts (10).

Preparation of Sulphydryl Groups — Sulphydryl groups in CNGRC-TNF were quantitated using Ellman’s reagent (Pierce) (11). Protein reduction and reoxidation were studied as follows: 3 mg of CNGRC-TNF in 0.5 ml of 0.1 M phosphate buffer, pH 8, was mixed with 10 mM β-mercaptoethanol (final concentration) and incubated for 30 min at room temperature. To separate the excess β-mercaptoethanol the product was loaded onto a PD10 column (Amersham) and eluted with 0.1 M phosphate buffer, pH 8. The CNGRC-TNF content in each fraction was monitored by measuring the absorbance at 280 nm. The pooled fractions (1.5 ml) contained 2.3 mg of protein. The amount of sulphydryl groups in the final product was quantitated using Ellman’s reagent after 1, 16, and 90 h of incubation at 4 °C. The CNGRC-TNF content in each fraction was monitored by measuring the absorbance at 280 nm. The pooled fractions (1.5 ml) contained 2.3 mg of protein. The amount of sulphydryl groups in the final product was quantitated using Ellman’s reagent. Each peptide was solvated with water in a periodic truncated octahedron large enough to contain the peptide and 1 nm of solvent on all sides. All solvent molecules within 0.15 nm of any peptide atom were removed. The resulting system was composed of 55 peptide atoms and 1474 water molecules for NoSS peptide and 53 peptide atoms plus 1081 water molecules for the SS peptide. The simple point charge water model was used (14). Each system was energy minimized with the steepest descent method for 1000 steps. Two simulations at the temperature of 300 K were performed and the temperature was maintained close to the intended value by weak coupling to an external temperature bath (15) with a coupling constant of τ = 0.05 ps. The peptide and the solvent were equilibrated separately to the temperature bath in both NoSS and SS simulations. The GROMOS96 force field was used (16). For water molecules the SETTLE algorithm (17) was used. A dielectric permittivity ε = 1, and a time step of 4 fs were used. A twin range cut-off radius was used for the calculation of the nonbonded interactions. The short-range cut-off radius was set to 0.8 nm and the long-range cut-off radius to 1.4 nm for both Coulombic and Lennard-Jones interactions. The cut-off values are the same as those used for the GROMOS96 force field parameterization used (16). Interactions within the short-range cut-off and the pair lists were updated every 5th time step. All atoms were given an initial velocity obtained from a Maxwellian distribution at the desired initial temperature. All the simulations were performed by 100-ps MD run. Then a 300-ps MD simulation was performed on each peptide in number of particles-volumes-temperature conditions. Cluster analysis was performed using the gromos method (18): the number of neighbors of one structure using a root mean squared deviation (RMSD)-based cut-off of 0.07 nm was counted; the structure with the largest number of neighbors with all its neighbors were taken as a cluster and eliminated from the pool of clusters. The procedure was then repeated for the remaining structures in the pool. All the MD runs and the analysis of the trajectories were performed using the GROMACS software package (19, 20). Graphical molecular models were generated using VMD software package (21).

RESULTS

Binding of CNGRC and GNGRG to CD13 — The CNGRC motif binds a CD13 isoform selectively expressed by tumor-associated vessels and recognized by the anti-CD13 mAb WM15 (7). To verify this hypothesis that the GNGRG motif can recognize this receptor also, we have studied the effect of CNGRC and GNGRG peptides on the binding of mAb (WM15) to the tumor vasculature, by immunohistochemistry. To this aim we tested the binding of mAb WM15 solutions containing various concentrations of each peptide (120, 60, 30, and 15 μg/ml) to renal cell carcinoma tissue sections. As expected mAb WM15 recognized an antigen expressed within the endothelial lining of tumor vessels (Fig. 1A). In contrast, no staining was observed with an irrelevant isotype-matched control antibody (not shown). When the sections were incubated with mAb WM15 in the presence of 120 μg/ml CNGRC or GNGRG peptides the staining of vessels was markedly reduced (Fig. 1, B and C). Of note, experiments carried out with lower peptide concentrations showed that the competitive activity of GNGRG was 2–3 times lower then that of CNGRC (data not shown). A control RGGN peptide did not affect the staining at any dose tested (Fig. 1D). These results suggest that both CNGRC and GNGRG motifs recognize the CD13 receptor, albeit with different affinity.

Preparation and Characterization of Human CNGRC-TNF and GNGRG-TNF — To investigate the effect of cyclic constraints on the NGR motif targeting properties, we prepared CNGRC-TNF and GNGRG-TNF conjugates. A glycine spacer was interposed between TNF and the targeting peptide in both conjugates. In addition, we prepared human TNF lacking the targeting moiety.

Protein Characterization — The molecular mass, purity, and the identity of these products were determined by SDS-PAGE, Western blotting, and ESI-MS. As expected, reduced SDS-PAGE showed bands corresponding to about 18 kDa that were amidic groups were used as terminal groups for each peptide. Arg was considered to be protonated. Thus, the total charge of the peptide was +1. Each peptide was solvated with water in a periodic truncated octahedron large enough to contain the peptide and 1 nm of solvent on all sides. All solvent molecules within 0.15 nm of any peptide atom were removed. The resulting system was composed of 55 peptide atoms and 1474 water molecules for NoSS peptide and 53 peptide atoms plus 1081 water molecules for the SS peptide. The simple point charge water model was used (14). Each system was energy minimized with the steepest descent method for 1000 steps. Two simulations at the temperature of 300 K were performed and the temperature was maintained close to the intended value by weak coupling to an external temperature bath (15) with a coupling constant of τ = 0.05 ps. The peptide and the solvent were equilibrated separately to the temperature bath in both NoSS and SS simulations. The GROMOS96 force field was used (16). For water molecules the SETTLE algorithm (17) was used. A dielectric permittivity ε = 1, and a time step of 4 fs were used. A twin range cut-off radius was used for the calculation of the nonbonded interactions. The short-range cut-off radius was set to 0.8 nm and the long-range cut-off radius to 1.4 nm for both Coulombic and Lennard-Jones interactions. The cut-off values are the same as those used for the GROMOS96 force field parameterization used (16). Interactions within the short-range cut-off and the pair lists were updated every 5th time step. All atoms were given an initial velocity obtained from a Maxwellian distribution at the desired initial temperature. All the simulations were performed by 100-ps MD run. Then a 300-ps MD simulation was performed on each peptide in number of particles-volumes-temperature conditions. Cluster analysis was performed using the gromos method (18): the number of neighbors of one structure using a root mean squared deviation (RMSD)-based cut-off of 0.07 nm was counted; the structure with the largest number of neighbors with all its neighbors were taken as a cluster and eliminated from the pool of clusters. The procedure was then repeated for the remaining structures in the pool. All the MD runs and the analysis of the trajectories were performed using the GROMACS software package (19, 20). Graphical molecular models were generated using VMD software package (21).
recognized by anti-human TNF antibodies by Western blotting (not shown). ESI-MS analysis showed that the N-terminal methionine was removed in all products (Table I). The CNGRC domain and two in the TNF moiety. Sulfhydryl groups of CNGRC-TNF were undetectable by reaction with Ellman's reagent, indicating that after purification most cysteines were in a disulfide-bridged form and that the 4 cysteines of TNF conjugate contains 4 cysteines: two in the N-terminal CNGRC domain and two in the TNF moiety. Sulphydryl groups of CNGRC-TNF were undetectable by reaction with Ellman's reagent, indicating that after purification most cysteines were in an oxidative form. To evaluate the propensity of sulphydryl groups, in the CNGRC-TNF to form disulfide bonds in the presence of air, the product was reduced with β-mercaptoethanol and the kinetics of re-oxidation was studied using the Ellman's reagent. After reduction with 10 mM β-mercaptoethanol more than 80% of sulphydryls were reoxidized in 1 h (Fig. 2). These results indicate that the targeting moiety of CNGRC-TNF was in a disulfide-bridged form and that the 4 cysteines of CNGRC-TNF subunits have strong propensity to form disulfide bonds after reduction and reoxidation in the presence of air.

**In Vivo Anti-tumor Activity**—In a previous work (6) we showed that picogram doses of CNGRC-TNF administered to B16F1 tumor-bearing mice are sufficient to increase the anti-tumor activity of melphalan and that 10^6-10^7 greater doses of TNF are necessary to achieve comparable results. In other studies (7) we showed that the mechanism of action of CNGRC-TNF is based on vascular targeting and that a CD13 isoform expressed in tumor vessels is a critical receptor for the CNGRC domain of CNGRC-TNF. To compare the activity of CNGRC and GNGRG peptides as targeting ligands we measured the capability of different doses (in the picogram range) of CNGRC-TNF and GNGRG-TNF to enhance the anti-tumor activity of melphalan in the B16F1 mouse model. A single administration of melphalan (90 μg) or CNGRC-TNF alone, induced little or no effects (Fig. 3A). However, when both drugs were administered to mice the expected synergistic effect was observed (Fig. 3, B–D). In particular, synergism was observed with as little as 0.01–0.1 ng of CNGRC-TNF. When animals were treated with GNGRG-TNF, at least 10-fold more drug was necessary for inducing comparable anti-tumor effects (Fig. 3, B–D). These results indicate that although GNGRG is still capable of targeting TNF to tumors the CNGRC domain is much more efficient, suggesting that the presence of cyclic constraints may affect the targeting properties of NGR peptides.

**Molecular Dynamic Simulations of CNGRC Peptides With and Without Disulfide Bridge**—To investigate the effect of cyclic constraints on peptide structure, the dynamic and conformational behavior of CNGRC peptides with or without covalent bond between Cys^1 and Cys^5 (called “SS” and “NoSS”, respectively) were explored by analyzing the simulated trajectories. Simulations of the dynamic behavior of CNGRC peptides in the open and cyclic form in realistic conditions were performed for a time long enough to reach equilibrium conditions in which folding/unfolding events could be sampled multiple times for the open structure (NoSS peptide).

**Conformational State**—Fig. 4 shows the RMSD computed for the backbone atoms relative to the starting point of the simulations. The RMSD plots for NoSS (Fig. 4A) and SS (Fig. 4B) indicate that the conformation of these peptides, as a function of time, is different. In particular, the RMSD plot for NoSS shows the presence of two distinct conformational states. The state “A” with low RMSD (<0.25 nm) includes structures whose backbone geometry is similar to the extended conformation of the starting point after minimization, whereas the state “B” with high RMSD (>0.35 nm) collects structures from which a transition to a different set of conformations can be inferred. Interconversion between states A and B was observed several times during the simulation, indicating that folding/unfolding (closed to open conformational transitions) events in equilibrium were sampled by molecular dynamics generating an equilibrium distribution. The SS peptide behaved quite differently (Fig. 4B). The lack of distinct conformational states is likely because of the presence of the disulfide bond acting as a constraint that limits the flexibility of the backbone atoms.

**Gyration Radius**—Fig. 5 shows the radius of gyration of peptide backbone as a function of time. The radius of gyration is a measure of mass-weighted spatial distribution of the atoms in each peptide configuration: the obtained plots show that the conformational states A and B of NoSS have different values of gyration radius. Given that the lower values correspond to the existence time of state B (25–35, 60–80, 95–110, 150–186, and 192–220 ns), the state B appears to be characterized by a more compact (folded) distribution of the backbone atoms compared with state A. It is worth noting that global shape of the backbone of SS peptide is uniform and intermediate between the two states of NoSS peptide.

**Secondary Structure**—Fig. 6 shows the secondary structure content as a function of time, based on the Dictionary of Secondary Structure of Proteins algorithm (22). In the case of NoSS, the results show that the initial extended conformation rapidly (~300 ps) converts into a bend structure during the simulation. The bend structure can also frequently fold into a more ordered turn structure involving residues Gly^3-Arg^4. In the overall simulation this is the prevalent conformation although the geometry of this turn region can fluctuate into a bend state despite the absence of cyclic restraints. It is worth noting that the existence time of state B (Fig. 4A) corresponds to the existence time of the turn structure (25–35, 60–80, 95–110, 150–186, and 192–220 ns). This suggests that the folded (closed) state B is characterized by the presence of a β-turn in Gly^3-Arg^4 amino acids. Interestingly, a turn comprising residues Asn^2-Gly^3-Arg^4 is noted several times during the trajectory, although in a short range of time. The turn between Gly^3-Arg^4 is also present during the simulation of SS peptide...
**Table I**

Characterization of molecular mass and cytolytic activity of TNF, CNGRC-TNF, and GNGRG-TNF

| Product           | Molecular mass of subunits (Da) | Cytolytic activity<br>units/mg |
|-------------------|--------------------------------|---------------------------------|
|                   | Expected                      | Found                           |
|                   | With                           | Without                         |
| TNF               | 17481.9                       | 17350.7                         | 17349.0 ± 1.3 | 5.45 × 10^7 ± 3.1 |
| CNGRC-TNF         | 18070.5                       | 17939.4                         | 17937.6 ± 1.9 | 5.09 × 10^7 ± 0.3 |
| GNGRG-TNF         | 17980.4                       | 17849.2                         | 17843.1 ± 3.8 | 4.42 × 10^7 ± 1.3 |

* By ESI-MS.
* By standard cytolytic assay based on murine L-M cells.

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**Fig. 2.** Kinetics of sulfhydryl reoxidation in CNGRC-TNF after reduction with β-mercaptoethanol. Sulfhydryl groups in reduced CNGRC-TNF (1.66 mg/ml) were quantitated using Ellman’s reagent, 1, 16, and 90 h after the removal of reducing agent (β-mercaptoethanol) by gel filtration chromatography (see “Experimental Procedures”). Sulfhydryl groups at time 0 were calculated taking into account that each CNGRC-TNF subunit contains four cysteines.

(Fig. 6B). However, in this case the bend state is more frequent. Likely, the cyclic constraint of this peptide prevents the optimal turn geometry from being as frequently populated as in the NoSS peptide case.

**Hydrogen Bonds**—To further characterize the conformational features of each peptide we analyzed their hydrogen bond content as a function of time during the simulation. Hydrogen bonds were considered to exist if the distance between hydrogen atoms and acceptors was less than 0.25 nm and if the angle donor-hydrogen-acceptor was less than 60°. The results of this analysis show that the hydrogen bond between C = O of Cys^5^ and N-H of Asn^6^ and the interactions between C = O of Asn^2^ and N-H of Cys^5^, respectively, are present (25–35, 60–80, 95–110, 150–186, and 192–220 ns) in the NoSS simulation, whereas for the SS peptide these bonds are rarely found. The presence of those interactions is consistent with the secondary structure analysis, being present when the backbone of Gly^3^ and Arg^4^ is arranged in a β-turn or β-turn-like conformation (data not shown).

**Distances between Cysteines**—To analyze the propensity of the NoSS structure to mimic ring closure between Cys^1^ and Cys^5^, the distance between Ca atoms of two cysteines was monitored as a function of time. Fig. 7 shows that the distance between Ca atoms fluctuates from a minimum of 0.32 nm to a maximum of 1.26 nm, corresponding to the time of existence of the conformational states B and A, respectively. Higher distances do correspond to the unfolded extended conformation (state A) of the peptide, where the bend geometry is not optimal to allow intramolecular interactions, whereas lower values are characteristic for the folded state B. This behavior is paralleled by the distance between the sulfur atoms of Cys^1^ and Cys^5^; the minimum value for the S-S distance is 0.4 nm and the maximum is 1.5 nm. Noteworthy, in the case of SS peptide this distance is uniform and it can be superimposed to the folded state B of the NoSS peptide.

**Thermodynamic Stability**—Because the simulation of the NoSS peptide shows multiple interconversions between two states, the simulations can be considered in thermodynamic equilibrium. Thus, we can evaluate the relative thermodynamic stability between state A and state B of the NoSS peptide by evaluating the population ratio n_B/n_A. The population of state A (n_A) is computed counting the number of conformations having RMSD with respect to the extended starting structure less than 0.25 nm, whereas the population of state B (n_B) is computed counting the number of conformations having RMSD greater than 0.35 nm. The resulting population ratio n_B/n_A is 1.91, which corresponds to a ΔG = −1.608 kJ/mol at 300 K. Thus, state B appears to be favored in the simulation conditions, assuming a structure whose backbone conformational features are similar to the ones of the active SS peptide.

**Representative Conformations**—To define the representative conformations of the different states of the two peptides, cluster analysis of the backbone atoms using a RMSD cut-off of 0.07...
nm was performed on each of the trajectories. The resulting clusters for NoSS peptide were 215, whereas 34 clusters were found for SS peptide. Fig. 8A shows the representative conformation of the two most populated clusters of NoSS peptide. The representative conformation of the most populated cluster depicts all the features (in particular, the β-turn geometry involving Gly3 and Arg4) found for the folded state B (Fig. 8A, right), whereas the representative conformation of the second most populated cluster is an example of the unfolded state A (Fig. 8A, left). Fig. 8B (left and right) shows the central conformation of the first two most populated clusters of the SS peptide. It is worth noting that the number of clusters of the NoSS peptide is much higher than that of the SS peptide: this difference reflects the different conformational flexibility between the two peptides, proving that the open peptide is more flexible than the sulfur-bridged peptide, and can populate a wider range of conformations, including those whose geometry overlaps the one of the active SS peptide.

FIG. 4. RMSD as a function of time of backbone atoms of NoSS peptide (A) and SS peptide (B) in MD simulation with respect to the starting structure.

FIG. 5. Radius of gyration as a function of time of each structure in the simulation for peptide NoSS (A) and SS (B).

FIG. 6. Secondary structure content as a function of time, based on the Dictionary of Secondary Structure of Proteins algorithm; of NoSS peptide (A) and SS peptide (B).

FIG. 7. Distance between Cα atoms of Cys1 and Cys5 in NoSS peptide. The minimum (0.32 nm) and the maximum (1.26 nm) values correspond to the time of existence of conformational states B and A, respectively.

DISCUSSION

We have previously shown that coupling CNGRC to TNF increases the anti-tumor activity of this cytokine in animal models (5, 6). This effect was blocked by co-administration of free CNGRC peptide but not of CARAC, pointing to a targeting mechanism to an NGR receptor. Moreover, anti-CD13 antibodies inhibited most of the anti-tumor activity of CNGRC-TNF, suggesting that CD13 (aminopeptidase N) is an important NGR receptor (5). Whereas the functional importance of NGR residues within the CNGRC binding motif is well documented, the roles of flanking residues and disulfide formation are less clear. The selection of both linear and cyclic NGR peptides by in vivo panning of phage-display libraries suggests that NGR-flanking cysteines are not necessary for binding (3). For instance, the selection of the NGRAHA peptide from phage-display libraries suggests that deletion of Cys1 and replacement of Cys5 with Ala do not abolish the tumor homing properties of the NGR motif. Other studies showed that incorporation of linear peptides containing the NGR motif into Moloney murine
leukemia virus envelope “escort” proteins enhances retrovirus binding to human endothelial cells more efficiently than cyclic peptides (9). However, it is unclear from these studies whether the lower efficiency of cyclic peptides was related to a poor incorporation of the targeting motif into virions or to a lower targeting efficiency.

In this work we have investigated the structure-activity relationships of the CNGRC targeting ligand and the role of the disulfide bridge, using synthetic peptides and recombinant TNF derivatives with cyclic (CNGRC-TNF) and linear (GNGRG-TNF) NGR domains. In addition, to explore the dynamic behavior of cyclic and linear peptides, we performed molecular dynamic simulation of CNGRC peptides in the open and cyclic form.

Competitive binding experiments with synthetic peptides and anti-CD13 mAb WM15 showed that both CNGRC and GNGRG motives can interact with CD13 in tumor vessels. Moreover, in vitro experiments showed that both GNGRG-TNF and CNGRC-TNF can induce anti-tumor effects at picogram doses. Because microgram doses of TNF are necessary to induce anti-tumor effects in this model (5, 6), these results are in line with previous works showing that both cyclic and linear NGR peptides can act as tumor-targeting ligands. However, 2–3 times more GNGRG than CNGRC peptide was necessary to compete the binding of mAb WM15 to tumor vessels in renal cell carcinoma sections. Moreover, the anti-tumor activity of GNGRG-TNF was more than 1 order of magnitude lower than that of CNGRC-TNF. These results indicate that the cystine-flanking NGR residues are critical for both CD13 binding and targeting efficiency, albeit to a different extent. This cystine could play a direct role by contributing to the interaction with the NGR receptor, or an indirect role by stabilizing the conformation of the NGR motif. Although the first hypothesis cannot be excluded, the results of molecular dynamic simulations of CNGRC with or without the disulfide bridge (SS and NoSS peptides) strongly support the hypothesis that peptide cyclization is critical for stabilizing the structure of the NGR motif.

Molecular dynamic simulation showed that the most populated and most probable configuration for the linear NoSS peptide (corresponding to state B in the RMSD plot) is characterized by the presence of a folded β-turn, involving residues Gly³-Arg⁴, and a strong hydrogen bonding interaction between the backbone atoms of Asn² and Cys². This configuration is thermodynamically favored likely because of the presence of intramolecular interactions in the folded state of the NoSS peptide. The unfolded state of NoSS (state A) is less populated and displays a bend-like geometry for residues Gly³-Arg⁴ not sufficient to minimize the distance between Cα atoms of Cys¹ and Cys². The cyclic SS peptide, on the other hand, is also characterized by the presence of the bend geometry involving residues Gly³-Arg⁴. However, in this case, the defined β-turn pattern and hydrogen bond interactions were not observed, probably because of the cyclic constraints.

These observations allow some speculation on the bioactive conformation of NGR peptides. Given the strong correlation between the bent conformation of the SS peptide and biological activity of CNGRC-TNF, we speculate that the state B configuration of the linear peptide is the bioactive one. The turn propensity of the NGR sequence likely favors the formation of intramolecular stabilizing interactions (such as hydrogen bonds), leading the linear peptide to populate an ensemble of conformations that are likely those recognized by the NGR receptors. This may explain the finding that GNGRG can target TNF to tumors, albeit to a lower extent than CNGRC.

The lower targeting efficiency of GNGRG could depend on several additive factors. For example, the affinity of GNGRG for its receptor could be lower than that of CNGRC, because the linear peptide have to enter the turn-like conformation to be recognized by the receptor. In addition, the linear peptide could adapt more efficiently to the binding pockets of proteases and peptidases, because of its higher conformational flexibility and its propensity to populate different conformational states. Consequently, GNGRG could be degraded in vivo at a higher rate than CNGRC. Measurement of receptor binding affinities is necessary to assess the real contributions of these factors. Unfortunately, the CD13 isoform expressed by angiogenic vessels has not been characterized yet and we have been unable to identify an in vitro cell system expressing functional CD13 for receptor binding studies. In any case, our results suggest that the in vivo NGR-targeting efficiency, which could depend on many factors, correlates with a more rigid turn-like structure.

The behavior of the NGR sequence can be compared with that of the RGD integrin-binding motif (23). Also in this case, structure-activity relationship studies showed that a folded structure is necessary for activity. Although theoretical and NMR-based experimental studies showed the presence of turn geometry also in the RGD sequence, centered on the glycine residue, the extreme conformational freedom of RGD makes, in this case, the open structure more favorable (24, 25). For this reason, the presence of conformational constraints is necessary to obtain the optimal geometry for receptor recognition, e.g. in the binding of RGDW with platelet glycoprotein IIb-IIIa (26). The lower capability of RGD peptides to assume the folded conformations, compared with NGR sequences, is likely related to the presence of two charged groups instead of one, which force the peptide to assume an extended conformation to obtain optimal solvation of charged side chains, as shown by Karplus and co-workers (24). In contrast, in NGR peptides the free energy gain obtained with solvation of only one charged side chain is more favorable in the folded conformations (the solvent accessible surface of Arg⁴ is 2.0 nm² for state B and 1.4 nm² for state A), where the formation of intramolecular interactions may further contribute to stabilize the folded state.
In conclusion, the results of our in vivo experiments and equilibrium simulations suggest that the NGR-turn inducing sequence tend to preferentially populate the ordered folded structure (state B). This latter conformation can reasonably be considered the active one, i.e. the one having the conformational characteristics that are necessary for binding to the NGR receptor. The disulfide bridge between the cysteines flanking the NGR motif is likely critical for stabilizing this conformation and for increasing the tumor targeting efficiency.

Acknowledgments—We thank Dr F. Magni for performing ESI-MS analysis of NGR-TNF and Dr. G. Arrigoni for providing renal cell carcinoma tissue sections.

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Structure-Activity Relationships of Linear and Cyclic Peptides Containing the NGR Tumor-homing Motif
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J. Biol. Chem. 2002, 277:47891-47897.
doi: 10.1074/jbc.M207500200 originally published online October 7, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207500200

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