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ARTICLE

NMR characterization of the C-terminal tail of full-length RAGE in a membrane mimicking environment

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Abstract Targeting the receptor for the advanced glycation endproducts (RAGE) signalling has a potential for the prevention and treatment of several pathologies. Extracellular activation of RAGE triggers the interactions of the RAGE cytoplasmic tail with intracellular protein partners. Here the cytoplasmic tail of RAGE has been investigated by NMR as part of the full-length protein, in the presence of a membrane-mimicking environment. The isolated cytoplasmic tail has also been studied for comparison. The NMR spectra of the whole receptor show that some but not all residues belonging to the C-terminal region of the cytoplasmic tail have a large flexibility, while the membrane proximal region seems to be rigidly connected to the trans-membrane domain and ectodomains. The analysis indicates that the behavior of the cytoplasmic tail is strongly affected by its being part of the whole receptor. These results provide new insight towards the understanding of signal transduction by RAGE.

Keywords RAGE · Signal transduction · Membrane proteins · NMR spectroscopy

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Introduction

The receptor for advanced glycation endproducts (RAGE) is a multiligand receptor of the immunoglobulin superfamily present on the cell surface and involved in inflammation and immune responses (Schmidt et al. 2000; Neeper et al. 1992; Sparvero et al. 2009; Chavakis et al. 2004). RAGE consists of an N-terminal extracellular portion, comprising domains V, C1 and C2 (23–317), of a transmembrane helix (343–363), and of a short cytoplasmic tail (364–404). The latter plays a crucial role in the transduction of the RAGE-ligand interactions (Hudson et al. 2008; Rai et al. 2012; Sakaguchi et al. 2011), but the details of the molecular mechanism and the modulation of RAGE activation are still largely unrevealed. Sequence alignment shows that there is a high level of conservation between the cytoplasmic tails of the various species, and excludes any enzymatic activity for the C-terminal domain of RAGE. According to the amino acid composition and to the described functional roles, this short sequence has been divided into three distinct regions, one proximal to the cell membrane, rich of basic amino acids, a central fragment rich of acidic amino acids, and a low-conserved C-terminal region (Ishihara et al. 2003). Here, the cytoplasmic tail of RAGE has been investigated as part of the full-length receptor in the presence of a membrane-mimicking environment, and compared with the isolated peptide. The analysis indicates that the behavior of the cytoplasmic tail is strongly affected by its being tethered to the whole receptor.

Materials and methods

Preparation of the protein samples

The cytoplasmic domain of RAGE, including three residues belonging to the trans-membrane domain (I361-P404,

cytRAGE hereater), was amplified by PCR from full-length RAGE DNA (GenBank NM_001136) with primers containing 5′ Bam HI and 3′ Xho I restriction sites. Then DNA was subcloned into the pGEX 4T-1 vector in order to express the protein fused with glutatione S-transferase (GST) followed by a thrombin cleavage site. The vector was transformed in *E. coli* BL21(DE3) strain (Novagen) and cells were grown in LB media at 37 °C till an $OD_{600} \sim 0.7$ was reached. Then expression of the recombinant protein was induced by addition of 1 mM IPTG and growth was allowed for 5 h at 37 °C. Cells were harvested by centrifugation at 9,000*g* and lysed by sonication in ice in Tris 50 mM pH 8, PMSF 1 mM, DNase 0.02 mg/ml. Clarified lysate, obtained by centrifugation at 40,000*g*, was first purified on DEAE column (GE healthcare) equilibrated with lysis buffer and eluted with a 10 column volume linear gradient to Tris 50 mM, pH 8, NaCl 1 M. The GST-tag was removed by thrombin cleavage (1 unit for 1 mg of protein) incubated at room temperature for 5 h followed by separation over Superdex 16.60 75 (GE healthcare) with HEPES 10 mM, pH 7.2, NaCl 150 mM. Expression and purity of the protein sample were verified by SDS-PAGE in 17 % polyacrylamide stained with Coomassie brilliant blue R-250 against Protein marker. Samples of ^{15}N- and ^{15}N,^{13}C-enriched RAGE-cytoplasmic construct were produced as described above except for the use of M9 minimal media containing $^{15}(NH_4)_2SO_4$ and ^{13}C-glucose as the sole nitrogen and carbon source, respectively.

Full-length RAGE DNA (GenBank NM_001136) was subcloned into the pET15b vector in order to express the protein fused with a histidine-tag followed by a thrombin cleavage site. The vector was transformed in *E. coli* BL21(DE3)-C43 strain and cells were grown in LB media at 25 °C till an $OD_{600} \sim 0.7$ was reached. Then expression of the recombinant protein was induced by addition of 1 mM IPTG and growth was allowed overnight at 25 °C. Cells were harvested by centrifugation at 9000*g* and lysed by sonication in ice with Tris 20 mM NaCl 300 mM, PMSF 1 mM, DNase 0.02 mg/ml at pH 8. Then the protein, precipitated as inclusion bodies, was solubilized with a buffer containing Tris 20 mM NaCl 300 mM, Urea 8M, PMSF 1 mM, SDS 0.2 % at pH 8. The solubilized protein was first purified by size-exclusion chromatography on the HiLoad 26/60 Superdex 75 (GE healthcare) equilibrated and eluted with the same Tris buffer. Then the protein, bearing the His-tag, was loaded in a Ni Sepharose FF column and refolded by using a renaturation buffer containing Tris 20 mM, NaCl 300 mM, DPC 0.2 %, at pH 8 and eluted with imidazole 500 mM. The refolded protein was further purified on a DEAE column (GE healthcare) equilibrated with a buffer containing Tris 20 mM, NaCl 40 mM, DPC 0.25 at pH 8 and eluted with a 10 column volume linear gradient to Tris 20 mM, NaCl 500 mM, DPC 0.2 % at pH 8. Then the elution buffer was replaced with the final buffer containing Tris 20 mM, NaCl 300 mM, DPC 5.7 % at pH 8. Samples of ^{13}C- and ^{15}N-enriched full-length RAGE were produced as described above except for the use of M9 minimal media containing $^{15}(NH_4)_2SO_4$ and ^{13}C-glucose as the sole nitrogen and carbon source, respectively.

NMR measurements and protein assignment

The experiments for the sequence-specific assignment of the cytoplasmic domain of RAGE (0.2–0.3 mM in water buffer solution, 10 mM HEPES pH 7.2, 150 mM NaCl) were performed at the temperature of 298 K on Bruker AVANCE spectrometers operating at 900 and 700 MHz and equipped with triple resonance cryoprobes. The assignment of H^N, N, C', C^α, and C^β resonances was obtained by the analysis of a set of ^{1}H-detected (2D ^{1}H–^{15}N HSQC, 3D HNCA, 3D HNCO, 3D CBCA(CO)NH, 3D HNCACB) and ^{13}C-detected *protonless* (2D hCON, 2D hCACO, 2D hCBCACO) NMR experiments (Bermel et al. 2006). The aliphatic side-chain ^{1}H and ^{13}C resonances were assigned through the analysis of 3D (H)CCH-TOCSY spectrum at 700 MHz. All the assigned atoms are reported in Table S1 and Table S2. The experiments on the full-length RAGE (0.1 mM in water buffer solution 20 mM Tris pH 8, 300 mM NaCl, 20 mM $CaCl_2$), and on its isolated cytoplasmic domain in the presence of DPC micelles were acquired at 298 K on a Bruker AVANCE spectrometer operating at 900 MHz and equipped with a cryoprobe.

R_1, R_2 and NOE measurements

The experiments for measuring ^{15}N relaxation rates and ^{1}H-^{15}N NOE were acquired at 298 K on a 700 MHz Bruker Avance spectrometer on ^{15}N-enriched samples of the cytoplasmic domain and full-length RAGE at the concentration of 0.2 and 0.1 mM, respectively. The ^{15}N longitudinal relaxation rates (R_1) were measured by collecting a series of 2D ^{1}H–^{15}N HSQC spectra using a sequence modified to remove cross correlation effects during the relaxation delay and considering variable inversion recovery delays ranging between 20 and 1500 ms for the isolated cytoplasmic domain and between 20 ms and 2000 ms for the full-length RAGE, respectively (Kay et al. 1989; Barbato et al. 1992). The recycle delay was 4.0 s with an acquisition time of 81 ms for all the relaxation measurements. The ^{15}N transverse relaxation rates (R_2) were measured using a CPMG sequence (Kay et al. 1989; Peng and Wagner 1994) with a CPMG refocusing delay, τ_{CPMG}, of 450 μs and with the variable delays ranging from 16.96 to 750 ms for the isolated cytoplasmic domain and from 8.48 to 245.92 ms for the full-length receptor, respectively. Heteronuclear $^1H - {}^{15}N$ NOEs were measured with and

without ^{1}H saturation. The relaxation data are reported in Table S3 and S4.

The effects of DPC on the isolated cytoplasmic domain were evaluated by adding minute amounts of a concentrated DPC (dodecylphosphocholine) solution (400 mg/ml) to a sample of the protein at the concentration of 0.33 mM in water buffer solution with 10 % of D_2O. 2D ^{1}H–^{15}N HSQC spectra were recorded after each addition and on the final sample, where the DPC concentration as monomer, was 240 mM.

Results and discussion

The isolated cytoplasmic tail of RAGE including three residues of the trans-membrane domain (I361-P404, cytRAGE) was expressed and purified as ^{15}N- and ^{15}N–^{13}C-enriched protein. The 2D ^{1}H–^{15}N HSQC spectra of the peptide show a poor spreading of the signals, as commonly observed for unfolded proteins (Fig. 1a). The NMR analysis of the protein was performed by a combination of ^{1}H-detected and ^{13}C-detected experiments that enabled the assignment of all but six (Gly-359, Ser-360, Gln-367, Arg-368, Arg-369 and Glu-380) NH resonances (Fig. 2a). The incompleteness of the assignment was caused by the low sequence diversity and the severe overlap involving these few residues. Secondary structure prediction was carried out with the program TALOS+ (Shen et al. 2009) using the H^N, N, C′, C^{α}, H^{α} and C^{β} chemical shift values as input data. The prediction suggests a random-coil conformation for the whole sequence with all the residues experiencing high mobility. Further and more detailed information on the dynamics of cytRAGE were provided by relaxation data. In particular, the characterization of fast motions occurring on picosecond-nanosecond time scales was performed by exploiting the spin relaxation properties of the amide ^{15}N nuclei through R_1, R_2, and NOE experiments (see Fig. 3a, c, e). Random-coil polypeptides and flexible protein regions are characterized by fast local motions with NOE values below 0.5 or negative. In our construct of cytRAGE, that includes also three aminoacids of the transmembrane region and two of the thrombin cleavage site, the heteronuclear NOEs for all amino acids are close to zero or even negative at the C-terminal region (Fig. 3e). These NOE values indicate that all the isolated cytRAGE is highly flexible and unfolded in solution. The absence of the α-turn structure observed by Rai and co-workers at the membrane-proximal region of the cytoplasmic tail (Rai et al. 2012) is possibly related to the slightly different experimental conditions and/or the three additional residues that are present in this construct. This is an indication that the presence or absence of secondary structure elements in cytRAGE can be critically dependent on its immediate environment.

Information on the cytoplasmic tail of RAGE in a more realistic environment can be achieved by analyzing the whole receptor in the presence of a membrane mimicking media. DPC micelles are frequently used not only as membrane mimetics for the structural characterization of peptides and proteins by solution NMR but also to solubilize and purify membrane proteins in their native conformation. Therefore, the full-length RAGE was cloned, expressed and characterized by NMR. In the 2D ^{1}H–^{15}N TROSY-HSQC spectra, performed on ^{15}N-enriched samples of the receptor in the presence of DPC micelles, only few poorly spread peaks are readily detectable (Fig. 1b).

The comparison of the spectra of the full-length receptor with those of the isolated cytoplasmic tail shows that most of the few observed cross-peaks belong to the cytRAGE. More in detail, most of the cross-peaks in the spectra of the full-length receptor correspond to signals of the isolated cytRAGE, with negligible chemical shift variations. The absence in the spectra of the signals relative to the amino acids of the other domains of the receptor (V, C1, C2 and the transmembrane domain) is interpreted as due to excessive broadening arising from the high molecular weight of the DPC-bound full-length RAGE. Very broad, unresolved additional peaks can be seen by lowering the intensity threshold with respect to that used in Fig. 1b (as shown in Figure S1, panel B). Self-assembly into oligomeric specie(s), suggested by native polyacrylamide gel (Figure S2) can also contribute to the broadening. Oligomerization phenomena have been reported also for short constructs of the receptor such as sRAGE at concentrations larger than 1 mg/ml (Sàrkàny et al. 2011).

Sixteen cross-peaks in the spectra of the full-length RAGE have been reassigned with high confidence to the residues A375, E377, E381, E382, E383, E384, A386, E387, L388, E393, E395, A396, G397, E398, G402 and G403, respectively (Fig. 1b), while for other six resonances (R373, Q379, R385, N389, Q390, E392) the assignment is less certain. Few other cross peaks, present in the spectra of the receptor, could not be assigned. At the same time, the cross peaks belonging to I361, L362, W363, R365, R366, G370, N378, S391, S399, S400, T401, respectively, are not present in the spectra of the full receptor. A detailed classification of the residues belonging to cytRAGE in the full-length receptor based on assignment certainty and disappearance of the cross-peaks from the spectra, is shown in Fig. 2b. It is immediately apparent from the distribution of the assigned residues along the sequence that the membrane proximal region of cytRAGE behaves very differently from the rest of the cytoplasmic tail. In particular, the assigned peaks belong to the amino acids downstream of the cytRAGE region, that has been recently reported to form an α-turn structure in the isolated peptide (Rai et al. 2012). The small or negative ^{1}H–^{15}N-NOE values, the large R_1 and the small R_2 values (see Fig. 3, panels b, d, f) of the assigned signals with respect to the isolated cytosolic tail in water buffered solution

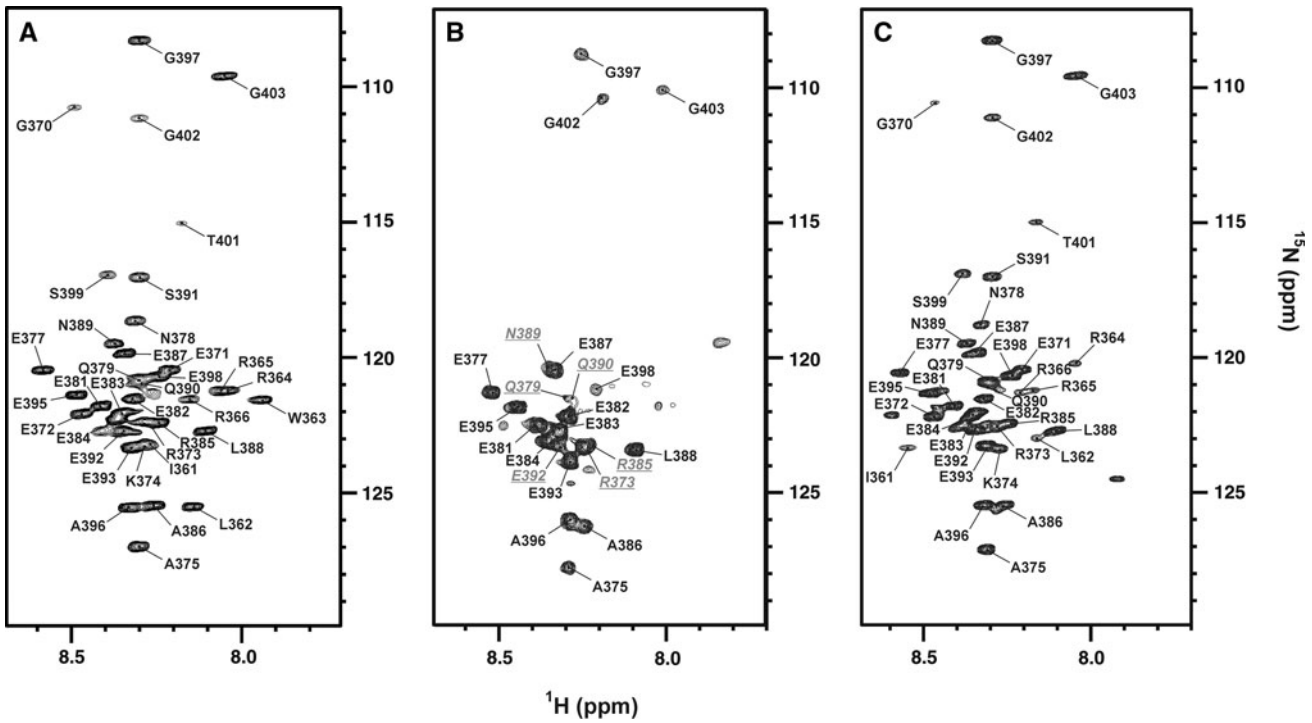

Fig. 1 **a** 2D ^{1}H–^{15}N HSQC spectra of cytRAGE in water buffer solution at 298 K. All resonances belonging to the isolated cytoplasmic tail but six have been assigned. **b** 2D ^{1}H–^{15}N TROSY-HSQC spectra of the full-length RAGE in the presence of DPC micelles at 298 K where sixteen signals belonging to the cytRAGE have been assigned with high confidence. For six additional aminoacids (*underlined* residues) a tentative assignment has been obtained. **c** 2D ^{1}H–^{15}N HSQC spectra of the isolated cytRAGE in the presence of DPC micelles at 298 K

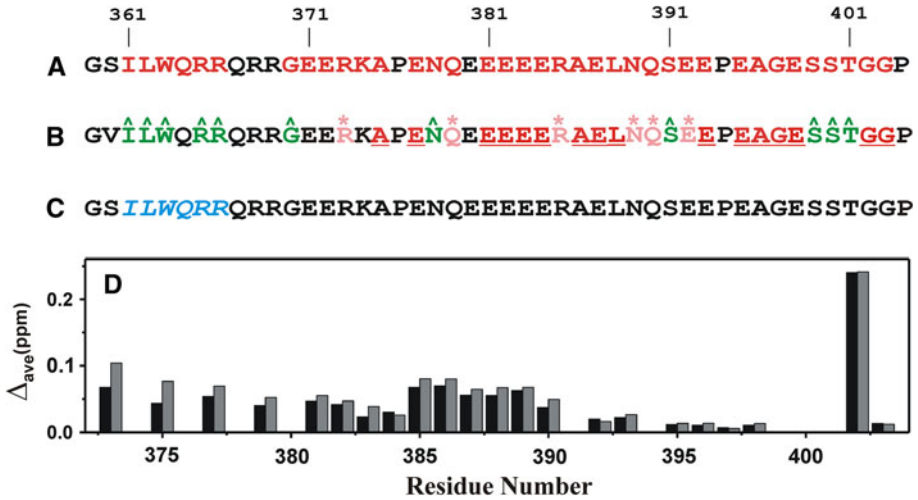

Fig. 2 Sequence of the expressed region of RAGE receptor (cytRAGE). The first two aminoacids (Gly–Ser in **a** and **c**) derive from the thrombin cleavage site, while the three contiguous residues (Ile–Leu–Trp) belong to the transmembrane domain. **a** In red, the residues of cytRAGE assigned in the 2D ^{1}H–^{15}N HSQC spectra when the polypeptide is dissolved in water buffer solution at 298 K; **b** in *red* (*underlined* characters), the residues of cytRAGE in the full-length receptor that are visible and reassigned with high confidence in the 2D ^{1}H–^{15}N HSQC spectra; in *pink* (indicated by *stars*), the visible residues for which the assignment is less certain; in green (indicated by *hats*), the residues whose peaks are not visible in the spectra of the full receptor and without any peak in the close proximity; in *black*, the residues that are not assigned even in the isolated cytRAGE, or those for which neither a tentative assignment, nor conclusive information on their disappearance can be provided. In the full-length receptor the serine, belonging to the thrombin cleavage site, is replaced by a valine; **c** in *cyan italics* fonts, the residues of the polypeptide undergoing chemical shift variations in the 2D ^{1}H–^{15}N HSQC spectra upon addition of DPC micelles; **d** plot of mean shift differences ($\Delta\delta = [(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{0.5}$) (Grzesiek et al. 1996) experienced by the residues of the cytoplasmic tail in the full-length receptor with respect to the isolated cytRAGE in presence (*black* columns) and in absence (*gray* columns) of DPC micelles

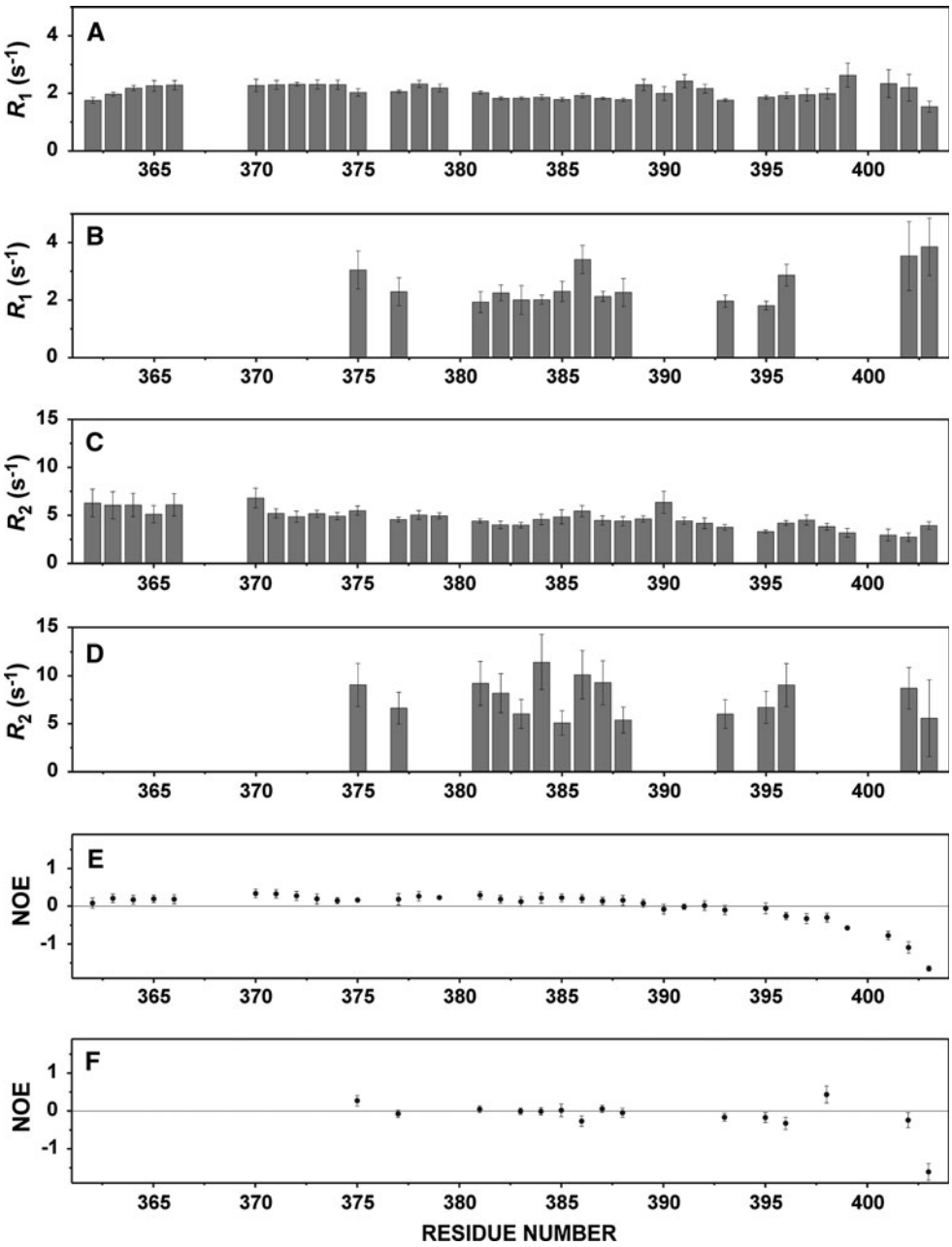

Fig. 3 Backbone ^{15}N relaxation data measured at 700 MHz and 298 K. R_1, R_2 and heteronuclear NOE values for the isolated cytRAGE are reported in panels **a**, **c** and **e**, respectively. R_1, R_2 and heteronuclear NOE values for the cytoplasmic tail in the full-length receptor are reported in panels **b**, **d** and **f**, respectively

indicates that the terminal region of cytRAGE in the full-length protein has a sizable degree of freedom. Conversely, the absence in the spectra of signals corresponding to the first six amino acids of the cytoplasmic tail suggests that the membrane proximal region of cytRAGE is probably rigidly held to the trans-membrane domain. This observation is consistent with the presence of an immobilized region at the N-terminus of the cytoplasmic tail that reduces the flexibility of this peptide-chain segment (Rai et al. 2012). The disappearance or shift observed for the other signals of cytRAGE (I361, L362, W363, Q364, R365, R366, G370, E371, E372, K374, N378, S391, S399, S400, T401) can be attributable to local interactions with the DPC micelles, when the cytoplasmic tail is tethered to the whole receptor. In particular, the increase of $^{1}H–^{15}N$-NOE values for the residues 398 and 402 and the relatively large change in the chemical shift for the latter residue in the full-length construct (see Fig. 2, panel d), seem to be consistent with the presence of transient interactions of the C-terminal region of the cytoplasmic tail with the membrane-mimicking micelles.

In an attempt to obtain additional structural information on the region of the cytoplasmic tail facing the membrane, the isolated cytRAGE was dissolved in the same membrane-mimicking environment used to characterize the full-

length receptor. The analysis of the 2D ^{1}H–^{15}N HSQC spectra (Fig. 1c) shows that the residues belonging to the trans-membrane domain, plus three residues at the membrane proximal region, experience sizable chemical shift variations upon increasing the DPC concentration (see Fig. 2c). The interaction of this cytRAGE construct with the membrane-mimicking environment is probably driven by the three hydrophobic residues, I361, L362, and W363 present in the N-terminal of cytRAGE and was not observed in a shorter construct (Rai et al. 2012). Conversely, the absent or negligible chemical shift variations observed for the residues at the C-terminal end indicates that this protein region does not interact appreciably with the micelles. Therefore, the addition of DPC micelles cannot reproduce in the isolated cytRAGE the effects observed in the full-length receptor, since all residues remain visible even in the presence of DPC micelles and none of the residues in the C-terminal region is shifted or disappears. Collectively, the effect of the membrane-mimicking media on the peptide resonances thus indicates that the isolated construct might not be a representative model of the cytoplasmic tail tethered to the whole receptor on the cell membrane.

Concluding remarks and biological implications

Although the molecular details of the signal transduction are still missing, it is known that the activation of RAGE signalling involves the interaction of the cytoplasmic domain with intracellular partners. cytRAGE is reported to bind different intracellular proteins such as mDia-1(Rai et al. 2012), ERK-1/2 (Ishihara et al. 2003) and after phosphorylation by PKCξ, TIRAP and MyD88 (Sakaguchi et al. 2011). In particular, it has been pointed out that the intracellular partner mDia-1 binds the α-turn at the membrane proximal region of cytRAGE, while the phosphorylation site Ser391 in the flexible tail is reported to be critical in the recognition of TIRAP and MyD88 proteins. It is reasonable to assume that the heterogeneous structural and dynamic properties of the cytoplasmic tail in the full-length receptor are functional to its broad binding capability toward multiple partners.

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