Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 5, pp 817–820, 1997

PII: S0968-0896(97)00021-7
0968-0896/97 $17.00 + 0.00

Detection and Structural Characterization of Ras Oncoprotein–Inhibitors Complexes by Electrospray Mass Spectrometry

A. K. Ganguly,* B. N. Pramanik,* E. C. Huang, S. Liberles,† L. Heimark, Y. H. Liu, A. Tsarbopoulos, R. J. Doll, A. G. Taveras, S. Remiszewski, M. E. Snow, Y. S. Wang, B. Vibulbhan, D. Cesarz, J. E. Brown, J. del Rosario, L. James, P. Kirschmeier and V. Girijavallabhan

Schering–Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, U.S.A.

Abstract—MS based methodology employing electrospray ionization (ESI) is described for the detection of ternary complexes in which SCH 54292 or SCH 54341 and GDP are noncovalently bound to oncogenic ras protein. The observed molecular weights of 19,816 and 19,570 Da confirmed the presence of noncovalent complexes of ras–GDP–SCH 54292 and ras–GDP–SCH 54341, respectively. We have also performed selective chemical modification of lysine residues of the ras protein complex followed by enzymatic digestion and on-line LC–ESI MS peptide mapping to determine protein–drug binding topography. There was a good correlation between nucleotide exchange inhibition as determined by the enzyme assay and evidence of complex formation as determined by MS. © 1997 Elsevier Science Ltd.

Introduction

In general, drug discovery efforts involve the development of new compounds that will bind to disease-causing proteins in a noncovalent nature, thus preventing the cause of the disease. Recently, we have designed and synthesized inhibitors of the oncogenic ras protein and have shown that these compounds bind noncovalently with ras–GDP. Ras proteins play an important role in signaling cell growth and differentiation.[1] They exist in two interconvertible states, the inactive ras–GDP and the active ras–GTP. Mutant ras proteins have been implicated in a wide range of human tumors.[2] Thus, inhibition of GDP–GTP nucleotide exchange in tumorigenic ras could prevent continued cell growth and hence, could represent a novel treatment for cancer. We have recently discovered a series of compounds that inhibit the exchange of ras–GDP to ras–GTP, and our intention was to study the drug–ras–GDP complex by X-ray crystallography. However, numerous attempts to co-crystallize our lead compounds with ras–GDP were unsuccessful. Therefore, the development of other spectroscopic methods to study drug–protein interactions was undertaken.

In this report we wish to describe the use of electrospray ionization (ESI) mass spectrometry (MS) to screen potential ras protein inhibitors that bind noncovalently with ras–GDP. An important feature of this report is the use of selective chemical modification and on-line LC–ESI mass spectrometric peptide mapping to probe protein–drug interaction and determine the drug binding topography.

The first application of using ESI MS for the detection of protein noncovalent interactions was reported by Ganem et al.[3] in which the binding between FKBP (an immunosuppressive binding protein) and immunosuppressive agents (FK506 and rapamycin) were studied. In a short period of time the technique has experienced a dramatic success, and noncovalent complexes of enzyme–substrate,[4] enzyme–inhibitor,[5a] and a small oligonucleotide duplex[5b] have also been detected by ESI MS. We have been studying the noncovalent interaction of the ras protein with its ligands GDP and GTP using ESI MS,[6] and we have extended our studies to probe protein–protein noncovalent interaction with considerable success.[7] In the present communication we have used ESI MS for detecting two ternary complexes of ras–GDP with SCH 54292 and SCH 54341, and for identifying the drug–protein (SCH 54292–ras–GDP, SCH 54341–ras–GDP) binding sites. We have recently communicated[8] the use of NMR spectroscopy and molecular modeling for structural studies of the SCH 54292–ras–GDP complex.

Results and Discussion

ESI analysis of a solution containing ras–GDP (M_r 19,295) and SCH 54292 provided multiply charged signals with an average mass of 19,816 Da (Fig. 1), corresponding to a ternary, noncovalent complex of ras–GDP–SCH 54292, thus confirming the 1:1 stoichiometry of the ras–GDP:SCH 54292 complex.

†Summer Intern at SPRI in 1993 from Harvard University, Cambridge, MA 02138, U.S.A.

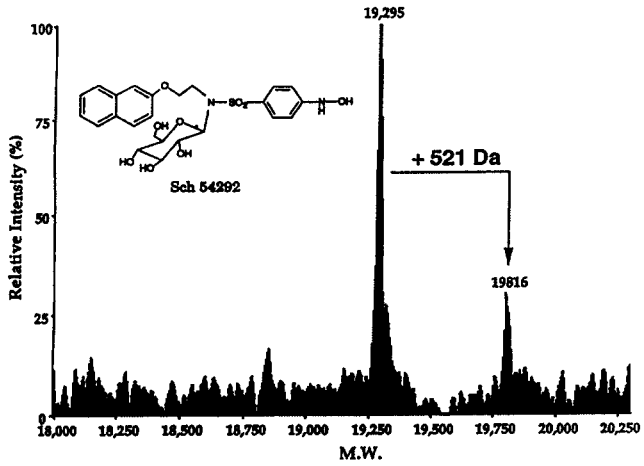

Figure 1. Deconvoluted electrospray mass spectrum of ras–GDP–SCH 54292 complex obtained on a Sciex API III triple quadrupole mass spectrometer. Aqueous solution of the complex (2 μg/mL in 2 mM NH_4OAc buffer; pH 5.8) was infused at 3 μL/min through the ion–spray interface. Several scans (10–20) from *m/z* 300 to 2400 at a scan rate of 2 s/scan were summed to yield the final profile spectrum prior to deconvolution.

The stability of the ras–GDP–SCH 54292 complex is highly dependent on the pH and the solvent system employed in the experiment, with the complex being more stable under near physiological conditions. Dissociation of the ras–GDP–SCH 54292 complex can be brought about either by adding organic solvent (10% methanol) or by lowering the pH of the aqueous solution of the complex. Under these conditions only ESI signals corresponding to apo-ras (M_r 18,852) are observed. These results correlate well with biological activity experiments, and lend support to the proposal that the gas-phase complex observed by ESI MS reflects the species present in solution under physiological conditions. Similarly, ESI analysis of the ras–GDP–SCH 54341 complex provided evidence for a 1:1 ras–GDP:SCH 54341 complex (M_r 19,570) (Fig. 2A). The intensity of that signal was enhanced by a factor of three when the ESI analysis was carried out under a lower infusion flow rate (3 μL/min vs 10 μL/min) and a lower orifice potential (30 V vs 80 V). It should be noted that ESI analysis of the biologically inactive nitro-phenyl analogue (SCH 53721) did not show any evidence of complexation (Fig. 2B).

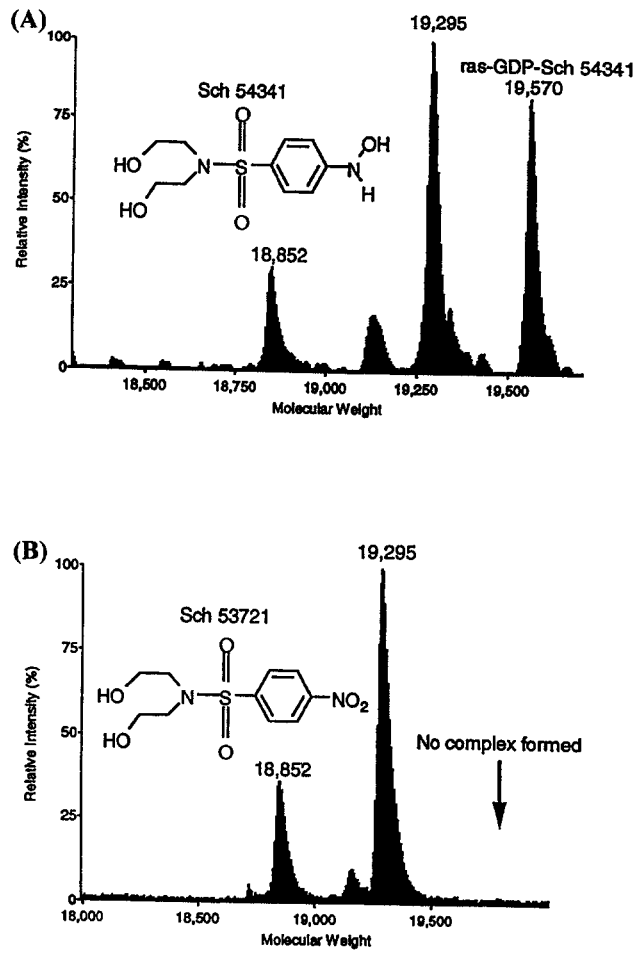

Figure 2. Deconvoluted electrospray mass spectrum of (A) ras–GDP–SCH 54341 and (B) ras–GDP–SCH 53721 complexes.

Information about the SCH 54292 binding site was obtained by carrying out covalent modification of the protein[9] followed by LC–ESI MS peptide mapping.[10] Under appropriate conditions, succinic anhydride reacts specifically with the ε-amino group of exposed lysine (Lys) residues on the surface of a protein. It was reasoned that Lys residues involved in SCH 54292 binding, or being adjacent to the binding site, may be sterically hindered by the bound drug and thereby will be protected from succinylation. Enzymatic digestion of

Table 1. Endoproteinase Lys-C peptides of ras protein with calculated and observed molecular weight values by LC–ESI MS

Position	Sequence	Peak Notation	Calculated $[M+H]^+$	LC/MS Observed $[M+H]^+$
1–5	MTEYK	L1	671.31	671
6–16	LVVVGAGGVGK	L2	955.59	956
17–42	SALTIQLIQNHFVDEY DPTIEDSYRK	L3	3095.53	3096
43–88	QVVIDGETCLLDILDT AGQEEYSAMRDQYMR TGEGFLCVFAINNTK	L4	5157.43	5157
89–101	SFEDIHQYREQIK	L5	1692.83	1693
102–104	RVK	L6	402.28	—[a]
105–117	DSDDVPMVLVGNK	L7	1388.67	1389
118–147	CDLAARTVESRQAQD LARSYGIPYIETSAK	L8	3312.66	3313
148–166	TRQGVEDAFYTLVREI RQH	L9	2318.20	2318

[a]Signal not observed by LC–ESI MS.

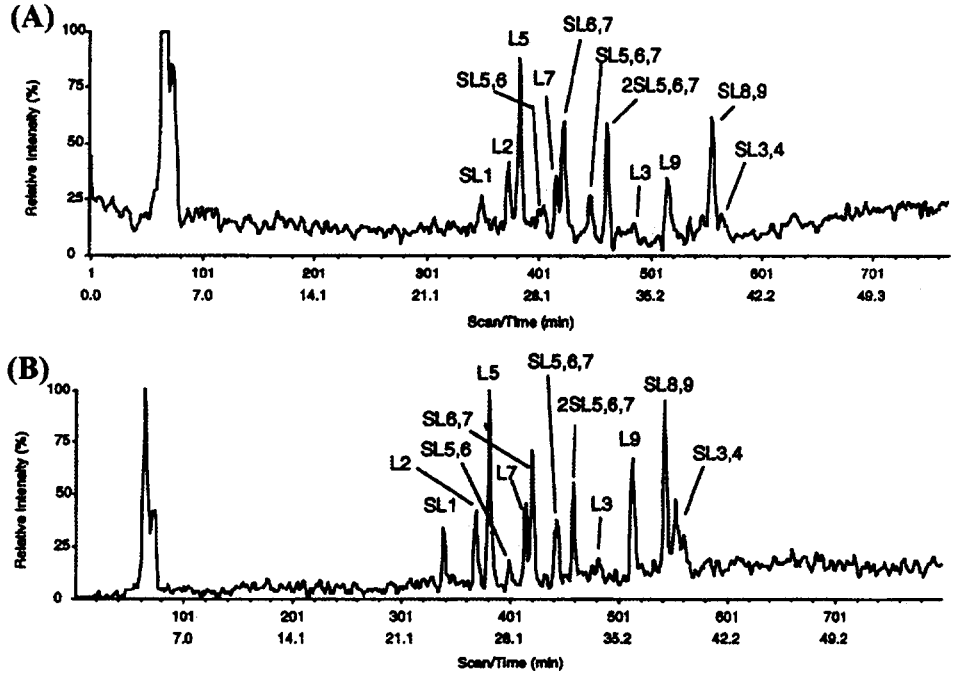

Figure 3. ESI LC–MS total ion chromatograms for (A) succinylated ras–GDP and (B) ras–GDP–SCH 54292 complexes following endoproteinase Lys-C digestion.

the succinylated protein and LC–MS analysis of the generated peptide fragments are then used to identify the unmodified Lys residues. Comparison of the succinate labeling patterns for apo-ras, ras–GDP and ras–GDP–SCH 54292 indicates which Lys residues are involved in SCH 54292 binding.

In each of the experiments, derivatization of the exposed Lys residues was performed by using a 10-fold molar excess of succinic anhydride in 25 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$. Following digestion of the derivatized proteins with endoproteinase Lys-C and peptide mapping by LC–MS analysis of the resulting peptide fragments, the identities of unmodified Lys residues were determined. Figures 3A and 3B show the respective total ion LC–MS chromatograms of succinylated ras–GDP and ras–GDP–SCH 54292 complexes following endoproteinase Lys-C digestion. The peaks are labeled to identify the digest fragment, with L1 referring to the first peptide fragment of the unlabeled ras protein comprising the *N*-terminus.

The observed mass values for each peptide fragment are shown in Table 1, along with the corresponding sequence of the resulting Lys-C peptides of ras.

Endoproteinase Lys-C cleaves proteins at the *C*-terminal end of a Lys but will not cleave at succinylated Lys residues. In that case the observed peptide fragment corresponds to two adjacent peptides plus a succinate group. For example, the label SL6,7 refers to succinylated fragment L6 plus L7 due to noncleavage at the succinylated Lys-104. It should be pointed out that some Lys residues are only partially succinylated (or partially protected from succinylation); therefore, a careful consideration of the relative intensities of all digest fragments is necessary in comparing the labeling patterns for the ras–GDP and ras–GDP–SCH 54292 complexes.

Using this approach it was determined that Lys-16, Lys-117, and Lys-147 are protected from succinylation in both ras–GDP and ras–GDP–SCH 54292 complexes. These residues are known from X-ray crystallographic data to be involved in GDP binding.[11] Lys-5 and Lys-88, located within the naturally folded protein, are also unlabeled in both complexes. The most obvious difference between chromatograms in Figures 3A and 3B is the increased intensity of the L5 peptide in Figure 3B suggesting protection of Lys-101 from succinylation. Considering all digest fragments involving Lys-101, if Lys-101 is protected from succinylation in the ras–GDP–SCH 54292 complex then the peak intensity of fragments L5 and SL6,7 (indicating cleavage at Lys-101) should increase relative to SL5,6 and 2SL5,6,7 (no cleavage at Lys-101). The ratio of L5 plus SL6,7 to SL5,6 plus 2SL5,6,7 is 1.7 for the ras–GDP complex but increases to 2.6 in the ras–GDP–SCH 54292 complex confirming that Lys-101 is protected from succinylation when SCH 54292 is bound to ras–GDP. On this basis, it was concluded that SCH 54292 must bind to ras–GDP near Lys-101 which is located on the underside of the cleft in the Switch II region. These mass spectral findings are consistent with results obtained for the ras–GDP–SCH 54292 complex by NMR and molecular modeling experiments.[8] Finally, it should be noted that these results indicate that the binding sites for SCH 54292 are different than those for the GDP/GTP nucleotides.

In conclusion, we have applied this ESI-based methodology to screen over 50 compounds for protein

inhibitors of ras–GDP. This methodology in combination with selective chemical derivatization and on-line LC–ESI MS mapping provided information on the drug–protein binding topography. Moreover, whenever there were ESI mass spectral signals indicating the inhibitor's noncovalent association with ras–GDP, there was also excellent correlation with the ensuing biological activity studies.

Experimental

The ras–GDP complex in 25 mM Tris, 5 mM $MgCl_2$ buffer (pH 7.4) was reacted for 5 min at room temperature with a 10-fold molar excess of succinic anhydride. The reaction pH was maintained between 7.0 and 7.5 by titration with 0.25 M KOH. After quenching the reaction with ammonium bicarbonate, the buffer was exchanged with 0.1 M ammonium bicarbonate, and the succinylated protein was then digested with endoproteinase Lys-C (with the pH adjusted to 8.5). The resulting peptide fragments were separated on a C18 microbore LC column using an ABI 140B dual-syringe pump. The mobile phase consisted of 0.1% TFA (A) and 0.1% TFA in acetonitrile (B), and a linear gradient of 5–75% B in 45 min at a flow rate of 40 μL/min was used for the separation. The resulting peptide fragments were analyzed on-line with a Sciex API III mass spectrometer as described previously.[12]

References

1. (a) Hall, A. *Science* **1990**, *249*, 635. (b) McCormick, F. *Cell* **1989**, *56*, 5.

2. Barbacid, M. *Eur. J. Clin. Invest.* **1990**, *20*, 225.

3. Ganem, B.; Li, Y.-T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 6294.

4. Ganem, B.; Li, Y.-T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 7818.

5. (a) Baca, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1992**, *114*, 3992. (b) Light-Wahl, K. J.; Springer, D. L.; Winger, B. E.; Edmonds, C. G.; Camp II, D. G.; Thrall, B. D.; Smith, R. D. *J. Am. Chem. Soc.* **1993**, *115*, 803.

6. (a) Ganguly, A. K.; Pramanik, B. N.; Tsarbopoulos, A.; Covey, T. R.; Huang, E. C.; Fuhrman, S. A. *J. Am. Chem. Soc.* **1992**, *114*, 6559. (b) Ganguly, A. K.; Pramanik, B. N.; Huang, E. C.; Tsarbopoulos, A.; Girijavallabhan, V. M.; Liberles, S. *Tetrahedron* **1993**, *49*, 7985.

7. Huang, E. C.; Pramanik, B. N.; Tsarbopoulos, A.; Reichert, P.; Ganguly, A. K.; Trotta, P. P.; Nagabhushan, T. L.; Covey, T. R. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 624.

8. Taveras, A. G.; Remiszewski, S.; Doll, R. J.; Cesarz, D.; del Rosario, J.; Vibulbhan, B.; Bauer, B.; Brown, J. E.; Carr, D.; Catino, J.; Evans, C. A.; Girijavallabhan, V.; Huang, E. C.; Heimark, L.; James, L.; Kirschmeier, P.; Liberles, S.; Nash, C.; Perkins, L.; Pramanik, B. N.; Senior, M. M.; Snow, M. E.; Tsarbopoulos, A.; Wang, Y.-S.; Ganguly, A. K.; Aust, R.; Brown, E.; Delisle, D.; Fuhrman, S.; Hendrickson, T.; Kissinger, C.; Love, R.; Sisson, W.; Villafranca, E.; Webber, S. E. *Bioorg. Med. Chem.* **1997**, *5*, 125.

9. Steiner, R. F.; Albaugh, S.; Fenselau, C.; Murphy, C.; Vestling, M. *Anal. Biochem.* **1991**, *196*, 120.

10. Huang, E. C.; Liberles, S.; Heimark, L.; Pramanik, B. N.; Ganguly, A. K. In *Proceedings of 42nd ASMS Conference on Mass Spectrometry and Allied Topics*, Santa Fe, NM, 1994.

11. Pai, E. F.; Krengel, U.; Petsko, G. A.; Goody, R. S.; Kabsch, W.; Wittinghofer, A. *EMBO J.* **1990**, *9*, 2351.

12. Tsarbopoulos, A.; Pramanik, B. N.; Nagabhushan, T. L.; Covey, T. R. *J. Mass Spectrom.* **1995**, *30*, 1752.

(*Received in U.S.A.* 5 *December* 1996)