CγH₂ of Met¹⁷⁴ Side Chain Is the Site of Covalent Attachment of a Substance P Analog Photoactivable in Position 5*

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Analogs of substance P (H-RPKPQQFFGLM-NH₂) incorporating a photoreactive para-benzoyl-l-phenylalanine (p-Bzl)Phe at position 4, 5, 6, 9, or 10 of the sequence have been synthesized and pharmaco logically characterized previously as full NK-1 receptor agonists. In this study we show that all analogs, [BAPA,p-(p-Bzl)Phe, Met(O₂)¹¹]SP also display high yields (40–70%) of NK-1 receptor photolabeling. To identify the site of photoinsertion in the receptor, covalent ligand/receptor complexes were digested with enzymes or chemically cleaved with cyanogen bromide and purified with streptavidin-coated magnetic beads before matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. Only the analog photoreactive at position 5/NK-1 receptor, with trypsin, endo-GluC and carboxypeptidase Y, led to the identification of the tripeptide [p-Bzl]Phe¹⁷⁴H₂SP¹⁷⁵ in the second extracellular loop of the hNK-1 receptor as the site of photoinsertion. Reaction of cyanogen bromide on the pentapeptide TMPSR did not yield the expected cleavage on the carboxylic side of methionine. The high precision of mass spectrometry analysis on the mass measured led us to determine that CγH₂ of Met¹⁷⁴ was the site of covalent linkage of the photoreactive substance P analog. Such an insertion (photoinserted ligand) on its CγH₂ renders methionine refractory to CNBr cleavage.

The substance P (H-RPKPQQFFGLM-NH₂) NK-1 receptor is a member of the class I family within the superfamly of G-protein-coupled receptors. The NK-1 receptor has been cloned in several species, among them the murine, the rat, and the human. The hNK-1 receptor is composed of 407 amino acids organized in 7 membrane-spanning segments. As for all other receptors from this superfamly, the molecular basis of the binding of the preferred endogenous ligand SP to the NK-1 receptor is far from being wel understood. Structure-activity relationships in SP (amino acid substitutions or modifications) (1) as well as in the NK-1 receptor (site-directed mutagenesis, construction of deleted or chimeric receptors) (2) have been studied extensively. Beside these studies, a complementary and more direct approach to identify the key points in the interaction between a peptide and a protein is photoaffinity labeling (3).

Several photoaffinity-labeling studies of the NK-1 receptor have already been reported (see Fig. 3) (4–10). All of these studies have used p-benzoyl-l-phenylalanine (p-Bzl)Phe as the photoactive probe that is activable at a wavelength of 365 nm. With analogs of SP incorporating (p-Bzl)Phe in the third or eighth position, Li et al. have shown that these two residues are in contact, respectively, with the N-terminal extracellular tail (residues 1–21) and residues 173–183 of the second extracellular loop of the murine NK-1 receptor (4). Using a photoreactive analog at position 8 of SP, Kage et al. reported the site of covalent attachment as Met¹⁸⁵ in the rat (5), whereas we identified Met¹⁷⁴ in the second extracellular loop of the human (6, 7) or the rat NK-1 receptor (7). During the course of this study, Bremer et al. have reported that photoinsertion of a photoreactive analog of SP at position 3 is located both within the segments 173–177 of the second extracellular loop and 11–21 of the N terminus of the rat NK-1 receptor (8). Li et al. have also identified Met¹⁷⁴ as the site of covalent attachment of a photoreactive analog of SP at position 4 (9).

To define further the binding site for SP within the NK-1 receptor, we have synthesized photoreactive analogs, [BAPA,p-(p-Bzl)Phe, Met(O₂)¹¹]SP, incorporating a biotinyl sulfone amino pentanoic acid (BAPA) derivative at the N terminus and the photoreactive amino acid (p-Bzl)Phe at position 4, 5, 6, 9, or 10 of the sequence of SP. We previously showed that the analog modified at position 7 has no affinity (µM) for the NK-1 receptor (6). The peptide analogs of SP photoreactive at position 4, 5, 6, 9, or 10 have high affinity for the two binding sites associated with the NK-1 receptor transfected in chinese hamster ovary cells and are potent agonists in activating both phosphatidylinositol hydrolysis and cAMP accumulation (11).

In this study, we show that these photoreactive analogs also give good yields of photolabeling of the NK-1 receptor transfected in chinese hamster ovary cells. We have used a methodology of photolabeling already described to identify the site of photoinsertion of analogs photolable in position 8, in the NK-1 receptor (6, 7). This methodology is based on the use of streptavidin-coated magnetic beads to purify the covalent ligand/receptor complex after enzymatic and/or chemical digestions and subsequent analysis by MALDI-TOF MS to identify the site of photoinsertion within the receptor (6, 7).

EXPERIMENTAL PROCEDURES

Materials—The photoreactive peptide analogs of SP have been synthesized and characterized previously (11). The MALDI-TOF mass spectrometer of the peptides gave the expected m/z of the protonated molecule (first isotope): 1890.90 expected, 1890.90 measured ([BAPA]², (p-Bzl)Phe¹¹, Met(O₂)¹¹)SP); 1859.90 expected, 1859.90 measured.

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(BAPA, (p-Bzl)PhE, Met(O2)11]SP, 1859.90 expected, 1860.02 measured (BAPA, (p-Bzl)PhE, Met(O2)11]SP); 1930.93 expected, 1931.05 measured (BAPA, (p-Bzl)PhE, Met(O2)11]SP); 1874.90 expected, 1874.77 measured (BAPA, (p-Bzl)PhE, Met(O2)11]SP). (11-3H[Pro9]SP (60 Ci/mmol) was synthesized at Commissariat à l'Energie Atomique (Saclay, France) according to Chassaing et al. (12). The Chinese hamster ovary cell clone expressing the hNK-1 receptor (6 pmol/mg) has already been described (6, 7). N-Tosyl-L-phenylalanine chloromethylketone-treated trypsin was from Sigma, endo-GluC and CBPY was from Boehringer, CNBr was from Calbiochem. Streptavidin-coated magnetic beads were obtained from Dynal.

Cell Culture and Membrane Preparation—Chinese hamster ovary cells expressing the hNK-1 receptor are cultured in Ham's F-12 medium supplemented with 10% (v/v) penicillin/streptomycin/2 mM glutamine. The production of the hNK-1 receptor was measured ([BAPA 0, (p-Bzl)Phe6, Met(O2)11]SP); 1930.93 expected, 1931.05 measured (BAPA, (p-Bzl)PhE, Met(O2)11]SP); 1874.90 expected, 1874.77 measured (BAPA, (p-Bzl)PhE, Met(O2)11]SP). ([11-3H][Pro9]SP (60 Ci/mmol) was synthesized at Commissariat à l'Energie Atomique (Saclay, France) according to Chassaing et al. (12). The Chinese hamster ovary cell clone expressing the hNK-1 receptor (6 pmol/mg) has already been described (6, 7). N-Tosyl-L-phenylalanine chloromethylketone-treated trypsin was from Sigma, endo-GluC and CBPY was from Boehringer, CNBr was from Calbiochem. Streptavidin-coated magnetic beads were obtained from Dynal.

Determination of the Yield of Photoaffinity Labeling—Membranes (10 μg of proteins) were incubated for 10 min at room temperature with the photoreactive analogs at a concentration equal to their affinity (K). Determination of competition experiments with [11-3H][Pro9]SP, i.e. 5 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, 20 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP. After irradiation, the covalent complex [BAPA, (p-Bzl)PhE, Met(O2)11]SP, 0.5 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, 13 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, and 18 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP in 100 μl of 50 mM Tris-Cl buffer pH 7.4 containing 1 mM EDTA, 10 mM MgCl2, 5 mM soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 400 μg/ml bovine serum albumin were purified with the magnetic beads and subjected either to further digestion with CBPY or MALDI-TOF MS analysis as described and were stored in 50 mM Tris-Cl pH 7.4, 1 mM EDTA, 10 mM MgCl2, 5 mg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 400 μg/ml bovine serum albumin as reference.

Identification of the Labeled Receptor Fragments by MALDI TOF MS Analysis—Covalently bound peptides were eluted from the magnetic beads with 3 μl of the MALDI matrix α-cyano-4-hydroxycinnamic acid in 4:1 (vol/vol) CH3CN/H2O (0.1% trifluoroacetic acid) as described previously (6, 13). After 10 min incubation, 1 μl of bead-free supernatant was desorbed on the sample holder for MALDI-TOF MS analysis. Mass spectra (averaged over 256 laser shots) were obtained in positive mode on a Voyager Elite (PerSeptive Biosystems) mass spectrometer in the reflector mode. Peptides for external calibration were deposited on the target close to the photolabeled samples. The deposit contains a few picomoles of neurotransmitter, and the accuracy in the mass range (800–10,000 Da) was estimated to be ±50 ppm. The samples gave a very low ion signal (resulting from a loss in peptide quantities because of the combined and repeated digestions and purification steps), the final mass spectrum was obtained from the average of 5–10 mass spectra (256 laser shots each). Typically, the amount of peptide on these targets is expected to be in the low-femtomole range. Peptide receptor domains corresponding to the mass peaks obtained were identified by using the Protein Analysis WorkSheet (PAWS) freelance edition (Proteomics, www.proteomics.com) and applied to the hNK-1 receptor using the different cleavages.

RESULTS

The synthesis and biological activity of all of these photoreactive analogs of SP have already been described (11). These analogs are potent competitors of [11-3H][Pro9]SP binding (K) value from 0.5 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP to 19 nm for [BAPA, (p-Bzl)PhE, Met(O2)11]SP to the NK-1 receptor more abundant binding site and all have agonist activity (11).

We first examined the efficiency of photolabeling with each of these photoreactive analogs of SP. The yield of photofinity labeling was determined to be 68 ± 1% for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, 54 ± 2% for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, and 57 ± 5% for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, 50 ± 10% for [BAPA, (p-Bzl)PhE, Met(O2)11]SP, and 46 ± 7% for [BAPA, (p-Bzl)PhE, Met(O2)11]SP of [BAPA, (p-Bzl)PhE, Met(O2)11]SP. These yields of photolabeling were sufficiently high to continue the analysis to identify the site of photoinsertion within the receptor.

After irradiation, the covalent complex [BAPA, (p-Bzl)PhE, Met(O2)11]SP/NK-1 receptor was digested for 15 h with trypsin. We observed by MALDI-TOF MS analysis of the tryptic or combined trypsin/endogluuc digestion that the analogs photoreactive at position 4, 5, 6, 9, or 10 are cleaved by the chymotrypsin-like activity derived from trypsin (from Sigma) as previously described (data not shown) (6, 7, 14). This cleavage even occurs with the sequencing-grade modified trypsin (from Promega) that should be extremely resistant to autolysis. For [BAPA, (p-Bzl)PhE, Met(O2)11]SP and [BAPA, (p-Bzl)PhE, Met(O2)11]SP, protonated molecules at m/z 1558.72 and 1527.74 (first isotope) were observed, respectively (shown only for the analog photolabile at position 5, Fig. 1a). They correspond to the N-terminal fragment 1–8 of these peptides. Similarly, for the analog [BAPA, (p-Bzl)PhE, Met(O2)11]SP, the N-terminal fragment 1–8 but also fragments 1–7, 1–6, and 1–5 were obtained (data not shown).

In the following, the given m/z ratio refer to that of the first isotope. With the exception of [BAPA, (p-Bzl)PhE, Met(O2)11]SP, all of the other photoreactive analogs only yielded peaks corresponding to the initial peptide or fragments of the initial peptide after photolabeling, enzymic cleavage, and purification via streptavidin-coated magnetic beads before MALDI-TOF MS analysis. With the analog [BAPA, (p-Bzl)PhE, Met(O2)11]SP and after trypsin treatment, reproducible MALDI mass spectra showed protonated molecules at m/z, 3031.23, 2868.25, and 2536.14 (data not shown, Table I). Controls (without photolabeling) confirmed that these ions are the main features of the photolabeling. Signals at m/z 3031.23 and 2868.25 can only correspond to the covalent attachment of the...
entire SP peptide analog to the receptor domains 168YSTTETMPSR177 (measured 1171.34 units, expected 1171.52 units) and 169STTETMPSR177 (measured 1008.39 units, expected 1008.46 units), respectively (Table I). The signal at m/z 2536.14 corresponds to the covalent attachment of the N-terminal fragment 1–8 of the photoreactive ligand to the receptor domain 169STTETMPSR177 (1008.45 measured, 1008.46 expected) (Table I). Subsequent cleavage of the tryptic fragments on magnetic beads with endo-GluC led to the identification in MALDI of new peptides and to the disappearance of the signal at m/z 3031.24 (Fig. 1b, Table I). Peaks at m/z 2450.1 and 2117.93 correspond to a unique receptor fragment, i.e. 173TMPSR177 (590.19 measured, 590.29 expected) (Table I). When using the sequencing-grade modified trypsin (from Promega), the same results were obtained. Subsequent digestion with CBPY (Fig. 1c) yielded new peptides in the MALDI mass spectra at m/z 2294.22, 1961.95, 1874.92, and 1814.83, corresponding to the receptor domains 173TMPSR176 (434.28 measured, 434.20 expected) and 173TMP175 (347.3 measured, 347.16 expected) linked to the entire sequence or to fragments 1–8 and 1–7 of the photoligand (Table I). Controls without UV irradiation only showed signals corresponding to the entire photoreactive peptide and the corresponding trypsin digestion-derived peptide fragments (Fig. 1a). Because the identified photolabeled do-

![Figure 1](http://www.jbc.org/)

**Fig. 1** Partial MALDI-TOF mass spectra obtained with [BAPA, (p-Bzl)Phe5, Met(O2)11]SP. a, mass spectra obtained without photolabeling and after combined trypsin, endo-GluC, and CBPY digestions. Asterisks refer to the photoreactive peptide [BAPA, (p-Bzl)Phe5, Met(O2)11]SP and its trypsin-digested fragments * (1–6), * (1–7), * (1–8). b, mass spectra obtained after photoling and combined trypsin and endo-GluC digestions. c, mass spectra obtained after photoling and combined trypsin, endo-GluC, and CBPY digestions. d, mass spectra after photoling, combined trypsin, and endo-GluC digestions and CNBr cleavage. The text in brackets indicate the domain of the receptor linked to the entire photoreactive peptide or to its fragments. The m/z values indicated on the figures correspond to the m/z of the first isotope.

| Treatment          | MH* measured | Covalent complex (receptor)- (photoligand) | MH* expected |
|--------------------|--------------|--------------------------------------------|--------------|
| Trypsin            | 3031.23      | (168–177)(1–11)                            | 3031.40      |
|                    | 2568.25      | (169–177)(1–11)                            | 2568.36      |
| Trypsin + endo-GluC| 2536.14      | (169–177)(1–8)                            | 2536.20      |
|                    | 2536.23      | (169–177)(1–8)                            | 2536.20      |
| Trypsin + endo-GluC + CBPY | 2450.20 | (173–177)(1–11)                            | 2450.19      |
|                    | 2294.22      | (173–176)(1–11)                            | 2294.10      |
| Trypsin + endo-GluC + CNBr | 1961.95 | (173–176)(1–8)                            | 1961.94      |
|                    | 1874.92      | (173–175)(1–8)                            | 1874.90      |
|                    | 1814.83      | (173–176)(1–7)                            | 1814.87      |
| Trypsin + endo-GluC + CNBr | 2450.13 | (173–177)(1–11)                            | 2450.19      |
|                    | 2402.05      | (173–177)(1–11) epoxide or ketone          | 2402.19      |
|                    | 2386.06      | (173–177)(1–11) ethylenic                 | 2386.19      |
|                    | 2117.95      | (173–177)(1–8)                            | 2118.03      |
|                    | 2070.07      | (173–177)(1–8) epoxide or ketone          | 2070.03      |
|                    | 2053.99      | (173–177)(1–8) ethylenic                 | 2054.03      |
main of the NK-1 receptor contains a methionyl residue, we carried out CNBr cleavage after digestion of the complex with trypsin/endo-GluC to restrict and to specify the site of photolabeling. MALDI MS analysis of the peptide obtained after trypsin, endo-GluC, and CNBr cleavage (Fig. 1d, Table I) led to the appearance of four new characteristic peaks at m/z 2402.05, 2386.06, 2070.07, and 2053.99 with a concomitant drop of the signals at m/z 2450.13 and 2117.95 observed in the corresponding experiments without CNBr treatment. These shifts of 48 units and 64 units, from both the entire photoreactive ligand and its N-terminal fragment linked to the entire photoreactive analog [BAPA<sub>5</sub>, (p-Bzl)Phe<sub>5</sub>, Met(O<sub>2</sub>)<sub>11</sub>]SP or its N-terminal fragment (1–8).

DISCUSSION

Several photolabeling studies have been carried out with the NK-1 receptor (Fig. 3) (4–10). In all cases, the photoactivable amino acid used is the same, i.e. para-benzoyl-L-phenylalanine. Combined results of these studies (Fig. 3) suggest that in SP, residue 8 contacts the methyl group on the sulfur of Met<sup>174</sup> (9), and residue 3 contacts both a segment extending from residues 173 to 177 and the extracellular N terminus within a segment extending from residues 11 to 21 (4, 8).

In this study, we have obtained a covalent and irreversible linkage of the hNK-1 receptor only with the analog of SP photoactivable in position 5 of the sequence. All other photoreactive peptide analogs were found, by MALDI-TOF MS analysis, either entirely or as digestion fragments but not linked to any receptor segment. This result was unexpected because these peptides have high affinity for the NK-1 receptor and because good yields of photolabeling could be determined in saturation experiments by comparing [<sup>3</sup>H][Pro<sup>9</sup>]SP maximal binding in the presence of these photoreactive analogs with or without irradiation. This protocol was used to circumvent potential difficulties to wash out the noncovalently bound photoreactive peptides. These results support the idea that a covalent and specific link had been created between the photoreactive peptides and the NK-1 receptor, whereas the possibility of noncovalent binding artifact can be dismissed. Therefore it is likely that some reversion of the photolabeling reaction occurred. The hypothesis of such reversion of the photolabeling reaction is acceptable, considering the specificity of the benzophenone chromophore. It has been well established (16) that the selectivity of hydrogen abstraction (X–H bond with X = C, O, N, or S) by the ketone oxygen of benzophenone when in solution depends on energy bond differences, resulting in a preferential abstraction of C–H bond. However, in the receptor-binding site, accessibility should also be taken into account, and the X–H bond strength is no longer the only parameter. After recombination of the radical, hemiacetal, aminal, or hemithioacetal can be formed with X = O, N, or S, respectively. These species are stable in nonaqueous environments (within the binding site), as ascertained by the photolabeling yields. However, during the subsequent steps (denaturation, enzymatic digestions, etc.), the three-dimensional structure of the receptor is lost, exposing these species to hydrolysis. This latter point could explain why the initial peptide (intact or enzymatically cleaved) is the only molecular species recovered by MALDI-TOF MS analysis.

On the contrary, a stable photoadduct has been formed with the analog [BAPA<sub>5</sub>, (p-Bzl)Phe<sub>5</sub>, Met(O<sub>2</sub>)<sub>11</sub>]SP. The photolabeling was specific for the NK-1 receptor because the covalent link was no longer observed in the presence of 10 µM [Pro<sup>9</sup>]SP (data not shown). Cleavage of the complex with trypsin and endo-GluC led to the identification by MALDI-TOF MS analysis of the pentapeptide 177<sup>TMPSR</sup> in the second extracellular loop of the NK-1 receptor as the site of photolabeling. Subsequent digestion of the pentapeptide with CBPY led to the tripeptide 177<sup>TMP</sup>. This tripeptide could not be further cleaved because, as reported, the cleavage of tripeptides is difficult and dipeptides are completely resistant to CBPY hydrolysis (17). To specify the site of photoininsertion within the tripeptide 177<sup>TMP</sup> in the NK-1 receptor, a chemical cleavage with CNBr was carried out on the pentapeptide 177<sup>TMPSR</sup> (because CBPY digestion is never complete, TMPSR was used instead of TMP for CNBr cleavage to have sufficient peptide quantity for mass spectrometry analysis). Products from CNBr cleavage were analyzed by MALDI-TOF MS. From CNBr cleavage we might have expected peaks corresponding to the photoreactive peptide linked either to 179<sup>TM</sup> (<sup>125</sup>IPMSR<sup>177</sup>), or -CH<sub>2</sub>ScN as previously shown for the analog photoactivable at position 8 of SP (5, 6). Unexpectedly, after CNBr cleavage, we only obtained a loss in mass of 48 units and 64 units that could not correspond to any amino acid. This observation led us to conclude that abnormal CNBr cleavage had probably occurred.
on the \( ^{172}\)TPSR\(^{177}\) branched peptide, a plausible explanation being the formation of a C–C bond between the photoreactive SP analog and the \( C_{34} H_{34} \) of methionine. With the use of a model compound, \((C_{6}H_{5})_{2}C(OH)CH_{2}SCH_{3}\) (15) we confirmed this hypothesis and showed that \( C_{7} H_{2} \) of the Met\(^{174}\) side chain is the site of photoinsertion (Fig. 2b). CNBr is widely used to cleave proteins at methionine residues (18). In this chemical cleavage, the sulfur atom of the Met side chain reacts with CNBr to lead to the sulfonium derivative (Fig. 2a). The Met side chain favors the intramolecular rearrangement of the sulfonium salt into the five-membered iminolactone. This iminolactone is then subsequently hydrolyzed to give an equilibrium between the homoserine lactone and its free acid form. In our case, the presence of the bulky group (photolinked SP) covalently linked to the \( C_{7} H_{2} \) of the methionyl residue must hamper the rearrangement of the sulfonium group in such a way that the iminolactone can no longer be produced. The rearrangement occurs between the diaryl \( \beta \)-alcohol and the sulfonium yielding the ethylenic and the epoxide or ketone derivatives with m/z values corresponding to the peaks experimentally observed (Figs. 1d and 2b). Formation of the ethylenic derivative of methionine side chain after \( S \)-carboxymethylation and exposure to acidic conditions of the granulocyte colony-stimulating factor has already been described (19). Moreover, a recent report on photocross-linking between \( \beta \)-amylloid peptides shows that benzophenone contacts the \( \delta \)-methyl group of a methionine side chain (20). It is also observed that methylation of methionine (to add more charge to the peptide and increase the number of fragment ions produced in MS/MS experiments) led spontaneously to \( \beta \)-elimination of the dimethyl thioether (\( \Delta m = -48 \) Da) after formation of the sulfonium ion, resulting in the formation of the ethylenic derivative between \( C_{6} \) and \( C_{7} \) of methionine side chain. However, in our study, the ethylenic bond is not located within the methionine side chain but between the \( C_{7} \) of Met\(^{174}\) and the carbone from benzophenone (ketone group). To our knowledge, this is the first example of such a CNBr reaction. This CNBr reaction on the covalent complex between \( ^{172}\)TPSR\(^{177}\) and the photoreactive analog has been repeated 5 times and always gave the same MALDI-TOF mass spectra. Therefore, \( C_{7} H_{2} \) of Met\(^{174}\) side chain is likely to be the primary (if not only) and more abundant site of photoinsertion of the SP analog photoreactive at position 5.

All of these results underline the power of mass spectrometry analysis compared with polyacrylamide gel electrophoresis to determine the mass of a peptide and the identity of the site of photoinsertion within a receptor at the atom level. For example, Behar et al. recently reported photolabeling of the PTH1 receptor with photoreactive and radiolabeled analogs of parathyroid hormone (21). Using CNBr cleavage of the covalent complex followed by electrophoresis and autoradiography, these authors concluded that one site of photolabeling could be either \( C_{7} H_{2} \) within a methionine or a different amino acid in the domain they identified within the parathyroid hormone receptor (21). In that latter case, the low resolution in mass determined by electrophoretic migration of the radioactive covalent complex did not permit to clearly identify the anchorage point in the receptor. Moreover, using this methodology, the authors (21) could not observe that covalent labeling on \( C_{7} H_{2} \) of methionine hampers CNBr cleavage as we did here using mass spectrometry analysis. Keeping in mind that thyl radicals are stabilized molecular species, \( C_{7} H_{2} \) and/or \( CH_{3} \) from methionine side chains represent attractive sites for photoinsertion of photoreactive ligands. Therefore, when such photolabeling is suspected, CNBr cleavage may then help to establish the exact localization of the site of photoinsertion. In this way, insertion on \( CH_{3} \) will yield the classical CNBr cleavage and give the mass difference (\( \Delta m = 72 \) as observed by mass spectrometry analysis but not electrophoretic migration) corresponding to addition of \( CH_{2}SCN \) on the initial photoreactive peptide (6). Photolabeling on \( C_{7} H_{2} \) of methionine side chain will lead to a loss in mass (\( \Delta m = 64 \) or 48 as observed by mass spectrometry analysis but not electrophoretic migration) corresponding to
the formation after CNBr treatment of the ethylenic and epoxide or ketone derivative as exemplified herein.

Taken together, the results from this study and previously published ones indicate that the binding points of SP within the NK-1 receptor seem to be almost exclusively located on the second extracellular loop, with the exception of the analog photoactivatable in position 5 that also interacts with the extracellular N terminus. The interaction between a ligand and a receptor is often studied from the ligand side by analyzing its potential bioactive conformation. It has previously been postulated from NMR studies that the bioactive conformation of SP is a helical structure between residues 3 and 8 (22). The fact that the same residue, Met\(^{174}\), is the site of interaction of photoactivatable amino acids at position 5 and 8 of SP supports the results obtained by NMR studies on the bioactive conformation of SP (22). Thus, the previously determined helical structure of SP is in complete accordance with results from photoactivatable studies.

Another concern is that most SP ligands containing the photoactivatable amino acid (\(p\)-Bzl)Phe and leading irreversible insertions to the NK-1 receptor cross-link into methionines. Indeed, McDonald et al. recently reported that the insertions sites of (\(p\)-Bzl)Phe\(^5\)-SP and (\(p\)-Bzl)Phe\(^8\)-SP into the NK-1 receptor are Met\(^{174}\) and Met\(^{181}\), respectivley (10). With the mutant M174A, the site of photoinsertion of (\(p\)-Bzl)Phe\(^4\)-SP becomes Met\(^{181}\) and not Ala\(^{174}\) (10). In a similar way, with the mutant M181A, (\(p\)-Bzl)Phe\(^5\)-SP photolinks Met\(^{174}\) and not Ala\(^{181}\) (10). When the two methionines are replaced, the photolabeling is no longer observed (10). Nevertheless, mutation of methionine to isoleucine (with a side chain length closer to that of Met than Ala, but without heteroatom) instead of alanine would have been better to study the role of the sulfur on benzophenone reactivity. However these observations may indicate a high preference of benzophenone for amino acids containing heteroatoms such as methionine. Purely chemical aspects have been reported nonexhaustively here are N-terminal domain (4, 31), loops E2 (32) or E3 (21), and transmembrane domains 3 (31), 6 (33), and 7 (34). These results suggest that all of these ligands and receptors interact in a unique way and that one model of recognition cannot be generalized to the whole G-protein-coupled receptor family.

What can be concluded here is that the identification of the site of photoinsertion of a ligand within a receptor can be solved at the atom level as exemplified herein with a SP analog photoactivatable in position 5 and the NK-1 receptor. This identification of the C.H. of the side chain as the site of covalent attachment of the SP analog was made possible by the use of mass spectrometry to analyze the digested covalent complex. This could not have been the case if we had used electrophoretic migration to determine the mass of the proteolytic fragments of the SP/NK-1 receptor complex.

REFERENCES

1. Quartara, L., and Maggi, C. A. (1997) Neuroptides 31, 537–563
2. Feng, T. M., Huang, R. C., Yu, H., Swain, C. J., Underwood, D., Cascieri, M. A., and Strader, C. D. (1995) Can. J. Physiol. Pharmacol. 73, 860–865
3. Dorman, G., and Prestwich, G. D. (2000) Trends Biotechnol. 18, 64–77
4. Li, Y.-M., Marnerakis, M., Stimson, E. R., and Maggio, J. E. (1995) J. Biol. Chem. 270, 1213–1220
5. Kage, R., Leeman, S. E., Krause, J. E., Costello, C. E., and Boyd, N. D. (1996) J. Biol. Chem. 271, 25797–25800
6. Girault, S., Sagan, T., Bolbach, G., Lavielle, S., and Chassaing, G. (1996) Eur. J. Biochem. 240, 215–222
7. Lequin, O., Bolbach, G., Frank, F., Convert, O., Chassaing, G., Lavielle, S., and Sagan, S. (2002) J. Biol. Chem., 277, 22386–22394
8. Bremer, A. A., Leeman, S. E., and Boyd, N. D. (2001) J. Biol. Chem. 276, 22857–22861
9. Li, H., McDonald, D. M., Hrnonski, X., Costello, C. E., Leeman, S. E., and Boyd, N. D. (2001) J. Biol. Chem. 276, 10589–10594
10. McDonald, D., Mierke, D. F., Li, H., Pellegrini, M., Sachais, B., Krause, J. E., Leeman, S. E., and Boyd, N. D. (2001) Biochemistry 40, 2530–2539
11. Sagan, E., Girault-Lagrange, S., Chassaing, G., Lavielle, S., and Sagan, S. (2002) J. Peptide Res. 59, 232–240
12. Chassaing, G., Lavielle, S., Julien, S., and Marquet, A. (1995) Tetrahedron Lett. 36, 623–626
13. Girault, S., Chassaing, G., Bolbach, G., Blais, J.-C., and Brunot, A. (1996) Anal. Chem. 68, 2122–2126
14. Keil-Dlouha, V., Zylber, N., Jove, N., and Keil, B. (1971) FEBS Lett. 16, 287–290
15. Sachon, E., Milcent, T., Sagan, S., Convert, O., Chassaing, G., and Lavielle, S. (2002) Tetrahedron Lett. 43, 7485–7489
16. Stone, P. G., and Cohen, S. G. (1981) J. Phys. Chem. 85, 1719–1725
17. Hayashi, R. (1977) Methods Enzymol. 47, 84–93
18. Gross, E. (1967) Methods Enzymol. 11, 238–255
19. Jones, M. D., Merreweather, L. A., Chogoton, C. L., and Lu, H. S. (1994) Anal. Biochem. 216, 135–146
20. Eganzyck, G. F., Greis, K. D., Stimson, E. R., and Maggio, J. E. (2001) Biochemistry 40, 11706–11714
21. Behar, V., Risello, A., Bhat, G., Rosenblatt, M., and Choyee, M. (2000) J. Biol. Chem. 275, 9–17
22. Chassaing, G., Convert, O., and Lavielle S. (1986) Eur. J. Biochem. 154, 77–85
23. Breton, C., Chellil, H., Kabbaj-Benmansour, M., Carnazzi, E., Seyer, R., Andrivet, P., Kabbaj, B., and Breton, C. (2001) J. Biol. Chem. 276, 18393–18401
24. Dong, M., Wang, Y., Hadar, M. E., Pinon, D. I., Holicky, E., and Miller, L. J. (1999) J. Biol. Chem. 274, 19161–19167
25. Ji, Z., Hadar, M., Henne, R. M., Patel, S. A., Lybrand, T. P., and Miller, L. J. (1997) J. Biol. Chem. 272, 24393–24401
26. Mille, J. S., Miuttenin, H. M., Bardin, D., Vlasses, J. M., Wimer-Mackin, S., Drata, E. A., Sunner, J., and Jesaitis, A. J. (1998) J. Biol. Chem. 273, 10428–10435
27. Zoffmann, S., Chollet, A., and Galzi, J. L. (2002) Mol. Pharmacol. 62, 729–736
28. Deng, X. Q., Delu, Y., Hadar, E. M., Holicky, E., L. Pinon, D. I., Lybrand, T. P., and Miller, L. J. (2001) J. Biol. Chem. 276, 4236–4244
29. Mouledous, L., Topham, C. M., Mazarguil, H., and Meunier, J. C. (2000) J. Biol. Chem. 275, 29268–29274
30. Breton, C., Chellil, H., Kabbaj-Benmansour, M., Carnazzi, E., Seyer, R., Phalipou, S., Morin, D., Durroux, T., Zengg, H., Barberis, C., and Moulel, E. (2001) J. Biol. Chem. 276, 20951–20954
31. Servant, G., Laporte, S., Leduc, R., Escher, E., and Guillemette, G. (1997) J. Biol. Chem. 272, 8653–8659
32. Keil-Dlouha, V., Risello, A., and Guillemette, G. (1996) Biochemistry 35, 13537–13544
33. Turk, J. W., Halsmus, T., Sullivan, N. L., Antonakis, K., and Le Breton, G. C. (2002) J. Biol. Chem. 277, 16781–16789
34. Laporte, S., Boucard, A., Servant, G., Leduc, R., and Escher, R. (1999) Mol. Endocrinol. 13, 578–586
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