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Solid-phase Synthesis and Cellular Localization of a C- and/or N-terminal Labelled Peptide

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Abstract: We report the solid-phase synthesis by the Fmoc strategy of a peptide containing a cysteamide group at its C-terminus. This peptide was subjected to further modifications including the linkage of fluorophores, namely lucifer yellow and coumarin respectively, at the C- and/or N-terminals. After incubation with living cultured cells these two probes were localized and it is concluded that the post-synthesis modifications can strongly modify the localization of the peptide.

Keywords: SPPS; labelled peptides; cellular localization; amphipathic peptide

INTRODUCTION

In order to elucidate the activity of multifunctional peptides which can act as carriers of biologically active materials and identify their potential targets, it is crucial to localize the peptides in biological material. Furthermore, the presence of proteases in biological media leads to the rapid degradation of all L-peptides and the fate of the various resulting peptide fragments may provide important information for the future design of resistant materials. One of the easiest to handle and one of the most efficient methods to examine these different aspects of peptide fate consists of the use of fluorescent labels which are covalently linked to the peptide. This approach may also provide information on the possible targets of compounds which could be carried by the peptide. Up to now, most of the investigations have dealt with side-chain labelled peptides, which makes them restricted to functional amino acids. However, as functional side chains are often involved in the biological role of peptides, it appeared that a peptide labelling that deals with C- or N-terminals or both, which participate less often in the biological activity, could provide an appropriate tool for the localization of these materials. Such a labelling, at least that of the C-terminal, can be easily achieved through the use of mercaptoamide peptides obtained after removal of disulphide linked peptide – resin which allows further chemical reactions dealing with the –SH group. This type of strategy allows the building up of the peptide by the conventional Fmoc-method [1, 2]. Subsequently the labelling can be made selectively after complete solid-phase peptide synthesis. Hence, the peptide can be labelled or not at its N-terminal when on the resin, while its C-terminal labelling occurs on one or the other peptide after cleavage from the resin.

In the present report we describe the synthesis of a peptide containing two domains; the first is hydrophobic, while the second is strongly hydrophilic and positively charged. Taken individually, they are expected to be localized in membrane and nucleus respectively. In order to determine the precise

Abbreviations: AEDI, aminoethyldithio 2-isobutyric acid; Cya, cysteamide; DOPG, dioleoylphophatidylcholine; HATU, O-(7-azabentriazole-1-yl)-1,1,3,3,tetramethyluronium hexafluorophosphate; MCA, 7-methoxy coumarin-2-acetic acid; NEM, *N*-ethyl morpholine; Pmc, *N*(2,2,5,7,8-pentamethyl) chroman-6-sulphonyl; TBTU, *O*-(1H-benzotriazol-1-yl)-*N,N,N'N'*=tetramethyl uronium tetrafluoroborate; TCEP, *Tris*(2-carboxyethyl)phosphine; Trt, triphenylmethyl.

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localization of the peptide or its degradation products we labelled the C- and N-terminals with lucifer yellow and coumarin respectively. Hence, the following peptides and conjugates were synthesized: X-G-A-L-F-L-G-W-L-G-A-A-G-S-T-M-G-A-R-K-K-K-R-K-V-Cya-X′ where X = H, Ac or methoxy coumarin and X′ = H or lucifer yellow. The chemical structure of the peptide was based on the association of two different sequences. The first one is issued from that of the fusion peptide of gp 41 [3, 4] which is known to interact with membranes [3] and where the Phe residues in position 7 is replaced by a tryptophan in order to introduce an intrinsic fluorescent probe in the peptide. The second sequence is hydrophilic and corresponds to that of the nuclear localization signal of the SV40 large T-antigen [5, 6]. This latter sequence was selected on the basis of a possible nuclear targeting of the peptide or of its linked probe. We describe here the behaviour of this material when incubated with biological material such as fibroblast cells.

MATERIALS AND METHODS

Chemicals

Purex grade dimethylformamide stored over 4 Å molecular sieves, HPLC grade acetonitrile, peptide synthesis grade piperidine and trifluroacetic acid were purchased from SDS (Peypin, France), 7-methoxycoumarin-4-acetic acid and DOPG were obtained from Sigma (St Quentin Fallavier, France) and lucifer yellow iodoacetamide dipotassium salt from Interchim Molecular Probe (Montluçon, France). TBTU, HOBT and Fmoc amino acids were obtained from Propeptide (Vert-le-Petit, France). The following protecting groups were used for side-chain protection: *t*Bu (Ser, Thr), Boc (Lys) and Pmc (Arg). AEDI–Expansion polymer (0.411 mmole AEDI/g) was prepared from the protected bifunctional disulphide handle (Boc-AEDI) and Expansin amino polymer as described previously. The AEDI content was calculated by amino acid analysis of taurine after direct performic oxidation of the AEDI support.

Analytical Procedures

Analytical HPLC was performed on a Waters HPLC (Milford, MA) using an Aquapore RP 300 column, C8, 7 μm, 220 × 4.6 mm (Brownlee Lab., Applied Biosystems, San José, CA) and semi-preparative HPLC was performed with a Nucleosil 300, C18, 5 μm column, 200 × 20, SFCC (Neuilly-Plaisance, France). Electrospray mass spectrometric analyses were carried out in the positive ion mode using a Trio 2000 VG Biotech Mass Spectrometer (Altringham, UK).

Amino acid analysis was performed on a High Performance Analyzer (Model 7300, Beckman Instruments, Fullerton, CA). For the analysis, the peptide (50 nmol) was hydrolysed in 0.2 ml HCl 5.7 N containing 0.9% (v/v) of phenol at 110°C for 23 and 46 h or for 24 h in 4N methanesulphonic acid for the tryptophan determination, under vacuum in sealed glass tubes.

Peptide Synthesis

The synthesis of the peptide was performed from 0.600 g of AEDI–Expansin (0.247 mmol AEDI) with a 9050 Pepsynthetizer Milligen (Millipore, UK) with the Fmoc/*t*Bu system as described previously [1,2] and according to manufacturer-specified protocols. Fmoc amino acids were activated by the addition of equimolar amounts of TBTU and HOBT diluted to 0.3 M with 5.5% NEM in DMF. Coupling was obtained by running this solution through the reaction column for a standard 45 min. Double couplings (45 min) were performed at crucial steps where, according to our own experience, a single coupling leads to a strong lowering of the yield and thus to a strong increase in difficulty at the purification step. These double couplings concern residues Thr_{14}, Ala_{17}, Arg_{18}, Lys_{21}, Arg_{22} and Val_{24}. Fmoc deprotection was achieved by percolating 20% piperidine in DMF through reaction column for 4.5 min at 3.8 ml/min for the first nine cycles and 1 min at 3.8 ml/min plus 7 min at 1.5 ml/min for the end of the synthesis. After deprotection of the terminal amino group, the side-chain protected peptidyl–resin was washed with DCM (4 ×), diethylether (4 ×) and dried under vacuum, yielding 1.2 g of material.

Acetylation

This procedure was performed as described in [2] on 0.6 g of the side-chain protected peptidyl–resin (0.099 mmol).

MCA Coupling

0.2 g of the side-chain protected peptidyl resin (0.033 mmol) was treated as above except that it was reacted for 1 h with 2 ml of preformed HOBT ester solution in DMF (HATU/HOBT/MCA/NEM; 10: 10: 10: 15 expressed in equivalents).

Removal of the Side-chain Protecting Groups

All three protected peptidyl–resins were deprotected for 4 h with 10 ml TFA/ethanedithiol/thioanisole/phenol/H_2O (94: 4: 2: 2: 2). The reactor content was then filtered out and washed with DCM (4 ×), DMF (4 ×), ethanol (3 ×), H_2O (3 ×) and finally with sodium acetate buffer 0.05 M, pH 4.5.

Peptide Release

Every deprotected peptidyl–resin was reacted with TCEP HCl (4 equiv. per mole of peptide on the polymer) in 15 ml of degassed 0.05 M sodium acetate buffer (pH 4.5)/DMF (1/1) for 16 h. The reactor content was then filtered out and washed with H_2O (3 ×) and ethanol (2 ×). The resulting crude peptide solution was evaporated and freeze dried. The yield of peptide release was over 99%.

Peptide Purification

After side-chain deprotection, the peptides were purified by semi-preparative HPLC in isocratic conditions (29, 35 and 38% acetonitrile in TFA 0.1% for the free, *N*-acetyl and *N*-coumaryl peptides respectively) at a flow rate of 5 ml/min. The purity of all collected fractions was checked by analytical HPLC and only the fractions leading to a single HPLC peak (right panel of Figure 1) were retained.

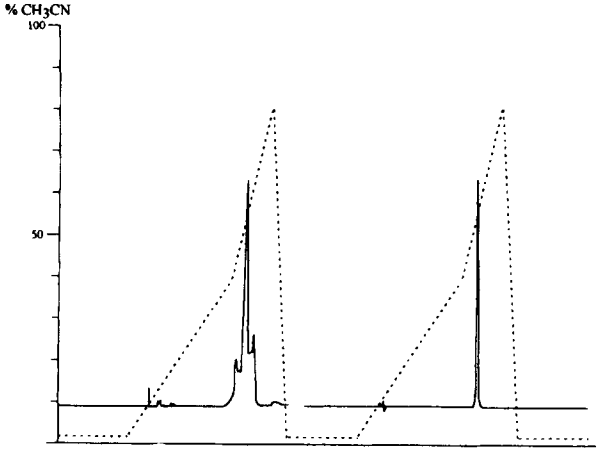


Figure 1 Reversed-phase HPLC analysis of the crude synthetic *N*-acetyl peptide (left panel) and of the same compound after purification (right panel). The flow rate was 1.5 ml/min and detection was performed at 280 nm.

Lucifer Yellow Coupling

5 mg of purified acetyl or coumaryl peptides in 2 ml of Hepes buffer (20 mM, pH 8.2) were added to 2.5 mg of lucifer yellow dissolved in 2 ml of the same buffer. After 30 min steering in the dark, the labelled peptides were recovered after HPLC purification at 29 and 26% acetonitrile respectively.

Cellular Localization Assays

Cells. Human fibroblasts (Hs-68) were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum as described previously. The cells were plated on glass coverslips and incubated for 3 min with the peptide dissolved in Hepes (100 mM) and then fixed with formalin (3.7%) for 5 min. The coverslips were rinsed with water, and mounted as described elsewhere [7].

Localization of the Peptide. The cells were observed by either confocal laser microscopy [7] or by fluorescence microscopy [8].

Spectroscopic Measurements

The fluorescence spectra were recorded on a spectrofluorimeter Spex-Fluorolog Model 1681 (Jobin-Yvon, Paris). The peptides were dissolved in Hepes 50 mM, pH 7.2. The excitation wavelength was 280 nm and the emission spectra were recorded in the 300–400 nm range for the non-labelled peptide and in the 300–600 nm range for the labelled ones.

The circular dichroism (CD) spectra were recorded on a Mark V dichrograph (Jobin-Yvon, Paris) using 1 mm thick quartz cells. Spectra in DOPG media were recorded after 1 h incubation of the peptide with DOPG vesicles which were prepared by a sonication procedure.

Infrared spectra were obtained on a Bruker (Wissembourg, France) IFS28. The samples were prepared by deposition of the peptide solutions or the peptide + lipid vesicles on a CaF_2 crystal. The spectra were recorded after evaporation of the solvent.

RESULTS AND DISCUSSION

Synthesis

The N-terminal free side-chain protected peptyl resin was obtained according to the standard Fmoc procedure (see Materials and methods) from 0.600 g

of AEDI–Expansin. The acetylation and 7-methoxy coumarin–4–acetic acid coupling were achieved on aliquots of the peptidyl resin. After HPLC analysis of crude peptides, all three were purified by semi-preparative HPLC (see Figure 1), leading to yields of 32.4, 37.2 and 21.75 (relative to the total amount of AEDI group on the initial support) for the three C-terminal free peptides, namely N-terminal free, N-terminal acetylated and N-terminal methoxycoumarin respectively. It must be mentioned that the elution volumes were respectively at 33.2, 37.2 and 41.5% acetonitrile (linear elution gradient of CH_3CN/TFA 0.1%) in accordance with the increase of hydrophobicity on going from the N-terminal free peptide to the coumarin derivative. All these peptides were identified by their mass spectra which were in line with the expected molecular weights: MH^+ at $m/z=2562$ (theoretical 2561), 2602 (theoretical 2603) and 2778 (theoretical 2776) respectively, as illustrated in Figure 2 for the coumaryl derivative. Also, the amino acid composition is in line with the expected one (Thr, 1 × 1.01; Ser, 1 × 0.99; Met, 1 × 0.96; Leu, 3 × 0.96; Gly, 5 × 0.985; Ala, 4 × 1.08; Val, 1 × 0.95; Phe, 1 × 1.00; Lys, 4 × 0.96; Arg, 2 × 1.00; and Trp, 1 × 0.99).

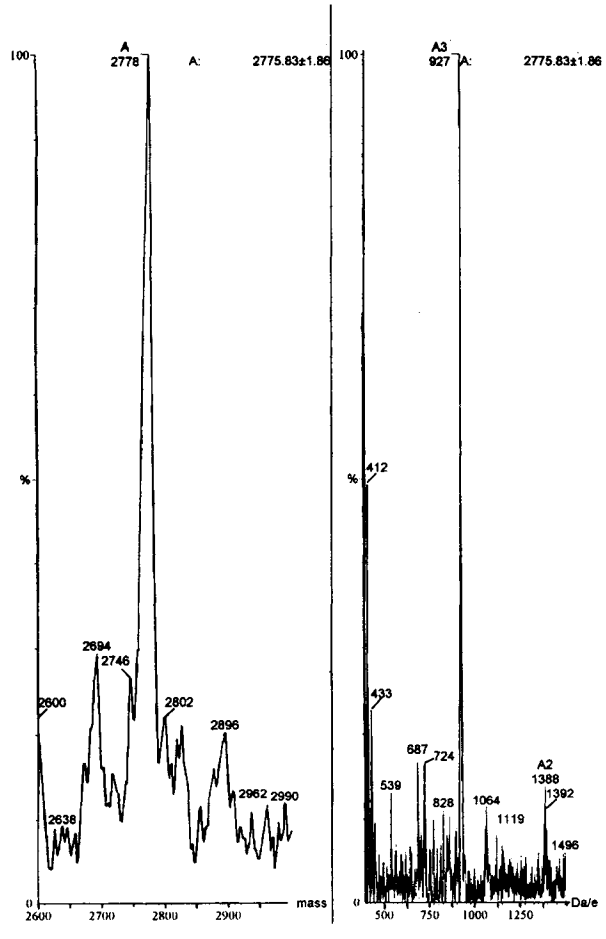


Figure 2 Electrospray ionization mass spectrum for the purified *N*-coumaryl peptide (right panel). The left panel shows the deconvoluted mass spectrum.

Hence, a single peptide synthesis provided three compounds differing by their N-terminal. However, the most important feature of these peptides lies in the fact that all of them carry, at their C-terminal, a cysteamide group which offers possibilities of adding functional partners or introducing further labelling without requiring a new and total synthesis of the peptide. Here, the work we describe will be restricted to the addition of a fluorescent label, namely lucifer yellow. After the couplings, the C-terminal labelled peptides were purified by HPLC with elution volumes at 29 and 26% CH_3CN leading to compounds with molecular weights MH^+ at $m/z=3127$ (theoretical 3126.5) and 3298 (theoretical 3299.5) as measured by mass spectrometry for the acetyl and coumaryl derivatives respectively.

Spectroscopic Characterizations of the Conjugates

In order to determine the tools which will be used for the identification of the cellular localization of the various fluorophores, it was important to describe the spectroscopic characteristics, especially through the identification of the fluorescence emission spectra in accordance with the excitation wavelengths of the confocal laser microscopy and/or of the fluorescence microscopy. Therefore, we undertook a fluorescence study of the labelled and unlabelled peptides. The various spectra are shown in Figure 3 and from examination of this figure it appears that the lucifer yellow derivatives are appropriate for confocal laser microscopy observations. Indeed, with an excitation wavelength at 425 nm, the lucifer yellow derivative leads to a spectrum with a fluorescence emission centred at 525 nm. As shown on Figure 3(a), this visible range emission depends only slightly on the excitation wavelength as when excited at 280 nm the emission maximum shifts to 540 nm. This figure also confirms the presence of a tryptophan residue characterized by a emission centred at 356 nm as found for the non-labelled peptide (Figure 3(b). The coumaryl derivatives are all characterized by fluorescence emissions centred at 390 nm when excited at 330 or 280 nm (Figure 3(c)–(e). These fluorescence characteristics indicate that the localization of the coumaryl chromophore cannot be achieved through confocal laser microscopy, but that

this fluorophore is compatible with conventional fluorescence microscopy. Therefore, from the above investigations it appears clear that the identification of the cellular localization of the coumaryl and lucifer yellow chromophores can be made using fluorescence microscopy while the observation by confocal laser microscopy will be restricted to the lucifer yellow containing compounds. Moreover, the low wavelength of the derivatives which are devoid of lucifer yellow (in the ultraviolet) significantly curtails their potential use for *in vivo* analysis since non-visible wavelengths frequently generate DNA damage.

Cellular Localizations of the Chromophores and Preliminary Structural Investigations

When incubated in presence of cells, the two chromophores, namely coumarin and lucifer yellow, in their non-peptidic forms, lead to spontaneous nuclear and perinuclear localizations respectively

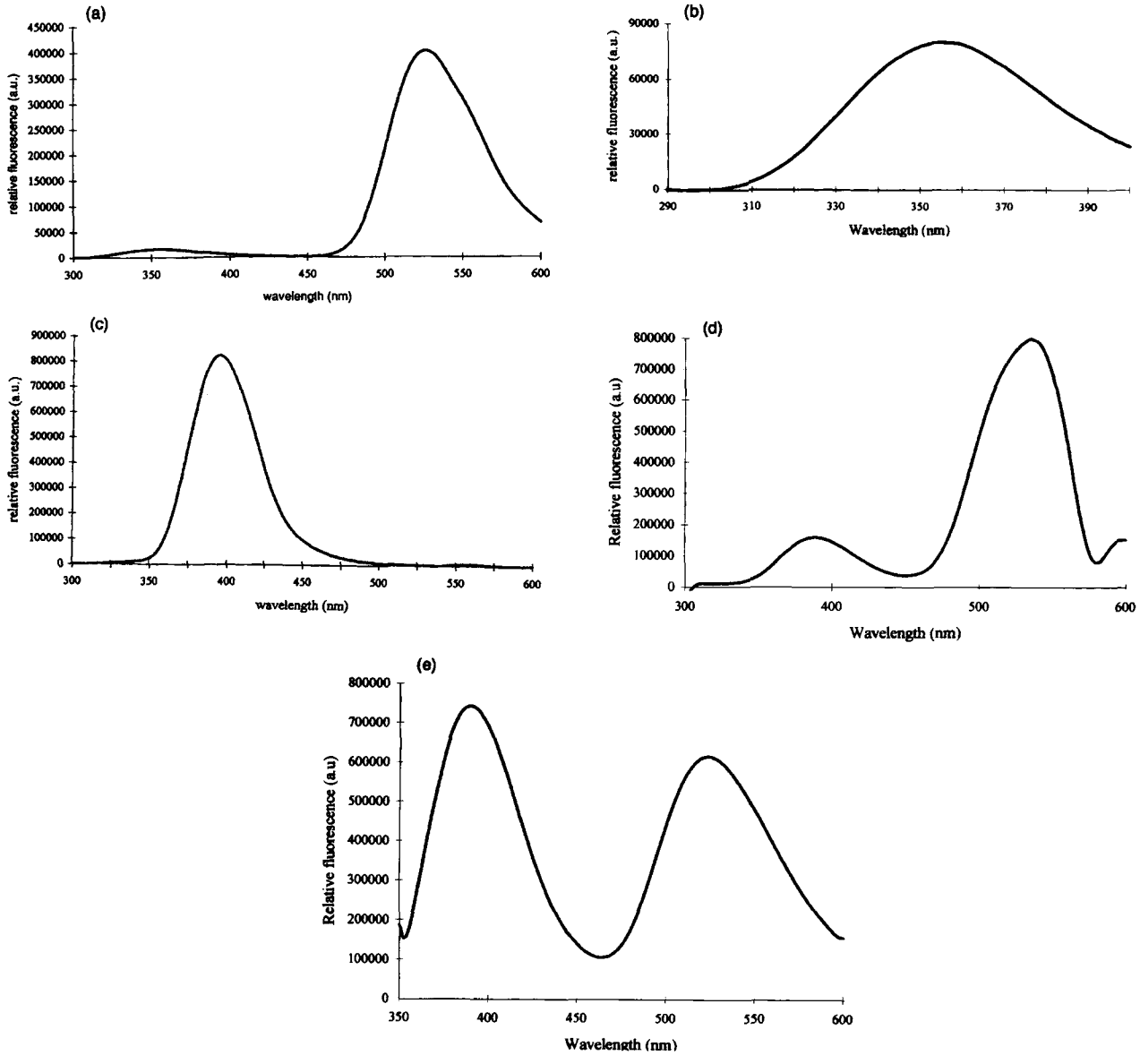
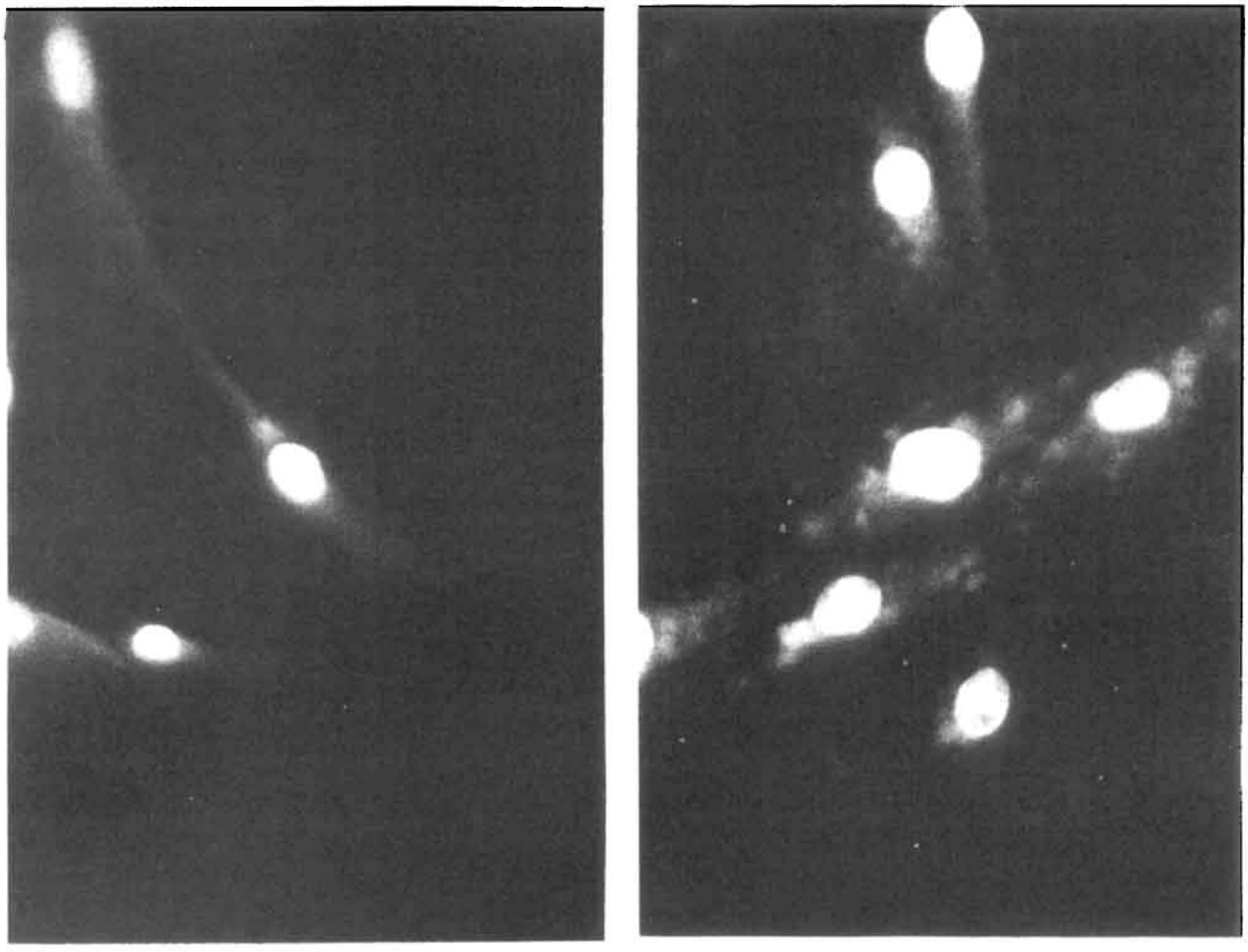


Figure 3 Fluorescence spectra of the various compounds described in this paper: (a) *N*-acetyl, *C*-lucifer yellow conjugate; excitation wavelength, 280 nm; (b) *N*-acetyl peptide; excitation wavelength, 280 nm; (c) *N*-coumaryl peptide; excitation wavelength, 280 nm; (d) *N*-coumaryl, *C*-lucifer yellow conjugate; excitation wavelength, 330 nm; (e) as for (d) but excitation wavelength, 280 nm.

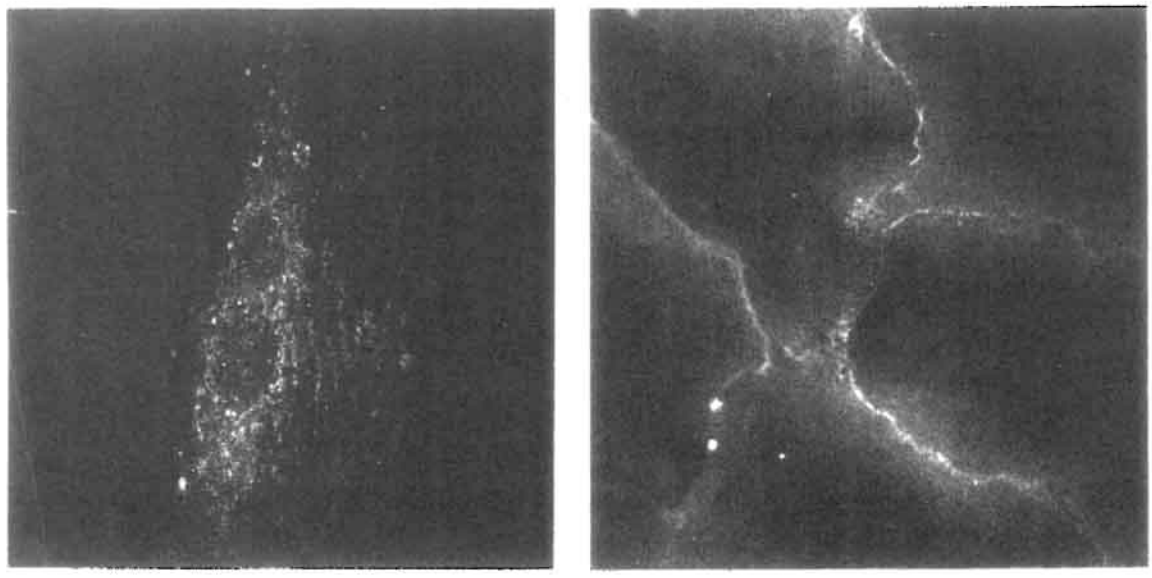
(a)

(b)



(c)

(d)



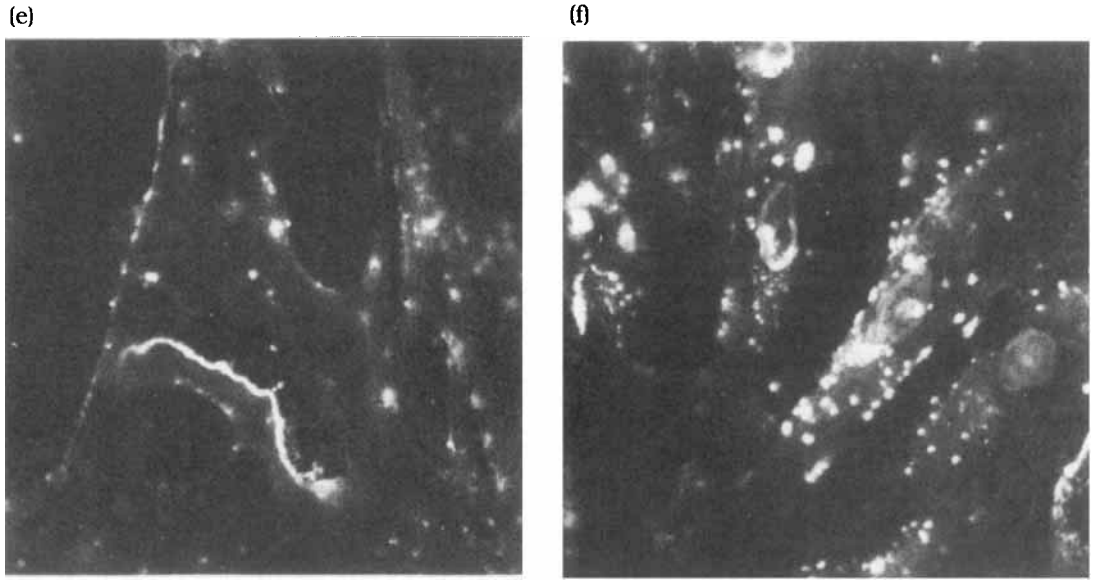


Figure 4 Visualization of the localizations of the various conjugates in fibroblast cells: (a) Free form of coumarin observed by fluorescence microscopy. The localization of the probe is nuclear. (b) *N*-coumaryl peptide observed by fluorescence microscopy. The label is principally found in the nucleus, but also in lyzosomes. (c) Free form of lucifer yellow observed by confocal laser microscopy. The probe shows a perinuclear localization. (d) *N*-acetyl, *C*-lucifer yellow conjugate observed by confocal laser microscopy. The label is principally localized in the plasmic membrane. (e) and (f) *N*-coumaryl, *C*-lucifer yellow conjugate observed by fluorescence microscopy. The localization is mainly lyzosomal but also nuclear and membrane-associated. An observation made by confocal laser microscopy (detection of the lucifer yellow probe) leads to very similar pictures.

(see Figure 4(a) and 4(c)). Their linkage to the peptides induces strong modifications of their cellular localization. Indeed, as shown on Figure 4, incubation of cells with the lucifer yellow conjugate leads to a localization of the chromophore in the membrane (Figure 4(d)) while in the case of the coumarin conjugate its localization is nuclear and vesicular or lysozomial (figure 4(b)). In the case of the double labelled peptide, this localization is more diffuse and most of the fluorescent material has a lysozomial localization (Figure 4(e) and 4(f).

These observations point out the roles of the various parts of the conjugates. It appears clear that the peptide, although it contains an NLS sequence [9,10], remains membrane-associated as exemplified by the lucifer yellow localization. As for the role of coumarin, its linkage to the peptide lowers the membrane anchoring of the peptide and induces its internalization, thus facilitating the action of proteases. Such a mechanism would account for a lysozomial localization of the peptide. These results point out the fact that chemical modification of peptides, which appear *a priori* minor, may strongly alter their properties and that investigations restricted to single labelling such as occurs for the N-terminal coumaryl conjugate would lead to erroneous conclusions with regard to the cellular localization of the peptide.

The behaviour described here has to be compared with several observations related to the fact that chemical modifications dealing with the C- or N-terminals of biologically active peptides may strongly modify their biological activity. However, for these latter, the results are mainly related to variations of binding affinities toward the targets of the peptides [11] and not, as in our case, to a modification of the localization of the peptide.

In order to confirm the various observed localizations, especially those that we propose are membrane-associated, and to try to elucidate the mechanism(s) leading to these localizations we undertook a physicochemical study including CD, fluorescence and also infrared spectroscopy. From the conformational point of view, it appears that the conjugates are extremely versatile molecules. For clarity reasons the preliminary CD results reported here will be restricted to the non-labelled peptide. These CD investigations indicate that the peptide is in a random coil form when in water or in buffer (phosphate or phosphate + NaCl) and that it under-

goes at least a partial random coil → α-helix transition upon addition of trifluoroethanol or acetonitrile to the medium (Figure 5). However, in the presence of lipid, DOPG vesicles in the present case, although some spectral deformations may occur because of the presence of vesicles, the CD spectrum rather suggests the presence of a β-structure. This latter conclusion, i.e. that the presence of lipids may induce the formation of β-structures, is confirmed by infrared observations made in the solid state. They reveal that lipid induces the appearance, in the amide I band region, of a contribution at 1630 cm^{-1} which is indicative of the presence of β-structures [12], while this contribution does not exist when the material is recovered from a solution in trifluoroethanol or water. In addition, fluorescence measurements provide information about the positioning of the peptide or of the conjugate with respect to the lipid. Indeed, when incubated with DOPG vesicles the fluorescence of the coumaryl fluorophore shows a lowering of the fluorescence intensity accompanied by a shift from 390 to 380 nm in its maximum which is in agreement with a lowering of the polarity of its environment. Also, the fluorescence of the tryptophan shows a shift from 355 to 333 nm, associated with an increase of the fluorescence intensity. All these observations account for a situation where the peptide is embedded in the lipid medium. Therefore, it can be stated that the ascribed membrane-associated localization really corresponds to a situation where the peptide is anchored in the lipid and not located at the membrane surface. As to the mechanism(s) which leads to these situations, owing to the strong conformational versatility of the peptide, it is not possible, at present, to decide whether the α-helix or the β-structure is responsible for the cellular penetration or internalization of the peptide.

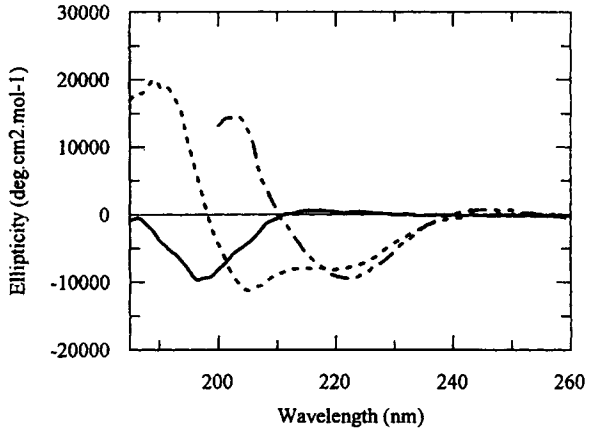


Figure 5 CD spectra of the *N*-acetyl peptide in different media (peptide concentration 0.1 mg/ml). ——— In water or phosphate buffer (pH 7.2). The spectrum is characteristic of a random coil form. - - - In water + 30% trifluoroethanol. It characterizes the presence of about 25% of a helical conformation as deduced from the ellipticity at 222 nm. — - - — Spectrum obtained after incubation of the peptide with DOPG vesicles at a lipid/peptide ratio of 30.

CONCLUSIONS

In conclusion, the present investigations confirm that mercaptoamide peptides are appropriate tools for investigations dealing with their cellular localization. This type of functionalization also provides the opportunity for post-synthesis chemical modifications such as the addition of fluorescent probes which allowed us to identify different localizations depending on the chemical structure of the conjugate. However, owing to the strong conformational versatility of the peptidic part which is able to undergo random coil → α-helix or random coil → β-structure transitions depending on the environment, it appears premature to propose a mechanism which could account for the various cellular localizations we have observed.

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