Fluorescent Indicators of Peptide Cleavage in the Trafficking Compartments of Living Cells: Peptides Site-Specifically Labeled with Two Dyes

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When cells are infected with viruses, they notify the immune system by presenting fragments of the virus proteins at the cell surface for detection by T cells. These proteins are digested in the cytoplasm, bound to the major histocompatibility complex I glycoprotein (MHC-I) in the endoplasmic reticulum, and transported to the cell surface. The peptides are cleaved to the precise lengths required for MHC-I binding and detection by T cells. We have developed fluorescent indicators to study the cleavage of peptides in living cells as they are transported from the endoplasmic reticulum to the Golgi apparatus. Specific viral peptides known to be "trimmed" prior to cell surface presentation were labeled with two dyes undergoing fluorescence resonance energy transfer (FRET). When these fluorescent peptides were proteolytically processed in living cells, FRET was halted, so that each labeled fragment and the intact peptide exhibited different fluorescence spectra. Such fluorescent cleavage indicators can be used to study a wide range of biological behaviors dependent on peptide or protein cleavage. However, labeling a peptide with two dyes at precise positions can present a major obstacle to using this technique. Here, we describe two approaches for preparing doubly labeled cleavage indicator peptides. These methods are accessible to researchers using standard laboratory techniques or, for more demanding applications, through cooperation with commercial or core peptide synthesis services using minor modifications of standard synthetic procedures. © 2000 Academic Press

Activation of cytotoxic T cells to clear intracellular pathogens and cancer cells requires peptide fragments of pathogen or cancer proteins to be presented at the cell surface, bound to the major histocompatibility complex I glycoprotein (MHC-I). Peptide cleavage specificities are important in determining which peptides are selected and in controlling the interactions of peptides with proteins that mediate trafficking between the endoplasmic reticulum (ER) and Golgi apparatus. Some viruses manipulate cleavage and trafficking to escape immune surveillance. Intracellular proteins are constitutively cleaved by the proteasome and then imported by TAP proteins into the lumen of the ER. There they bind to the heavy chain of MHC-I, which triggers binding of β_2-microglobulin to form a ternary complex. The ternary complex exits the ER and moves through the Golgi apparatus to the cell surface. When the peptide bound to MHC-I reaches the cell surface, it has been trimmed to an exact length, which depends on the particular MHC-I allele bound.

To examine how the trimming of peptides is coordinated with trafficking, we have produced fluorescent peptide cleavage indicators, peptides that bind MHC-I and change their fluorescence during cleavage. We have loaded these peptides into the cytoplasm of living cells and followed their changing localization and cleavage during ER-to-Golgi transport in real time. The cleavage indicators are based on fluorescence resonance energy transfer (FRET) between two dyes covalently attached to the peptide. One dye is on the portion of the peptide presented at

the cell surface and the other on a flanking sequence known to be cleaved prior to cell surface presentation. In the intact peptide, the two dyes undergo FRET, producing a unique fluorescence spectrum different from that of either cleavage product. Through appropriate selection of excitation and emission wavelengths during live cell imaging, the different species can be distinguished and the cleavage of the peptides can be followed (see Fig. 1). Here we detail two approaches that can be used to generate cleavage indicators, either by using solution-phase labeling of peptides obtained from commercial sources or by requesting that peptide synthesis facilities use minor modifications of their usual procedures to permit ready site-specific labeling by the investigator.

SELECTION OF DYE PAIRS FOR LIVE CELL FRET

FRET is exquisitely sensitive to dye separation, as the extent of energy transfer is inversely propor-

tional to separation raised to the sixth power. In approximate, practical terms, dyes usually used for live cell imaging show no FRET when separated by more than 100 Å, and FRET increases as they are brought closer together. Fluorescence is quenched when the dyes are within a few angstroms of one another. In cleavage indicators, the dyes show maximum FRET in intact peptide and no FRET after cleavage. Their placement on the intact peptide should be selected to bring them as close as possible without perturbing biological function, while preventing them from coming in contact with one another.

Dyes for live cell cleavage indicators must fluoresce at long wavelengths that do not overlap with cellular autofluorescence and do not require excitation at short, high-energy wavelengths that damage living cells. Optimum choices are dyes with excitation maxima greater than 450 nm, although shorter-wavelength dyes have been used. Other physical properties affecting the efficiency of FRET between two dyes are the overlap of the donor emission and acceptor excitation spectra, the extinction coeffi-
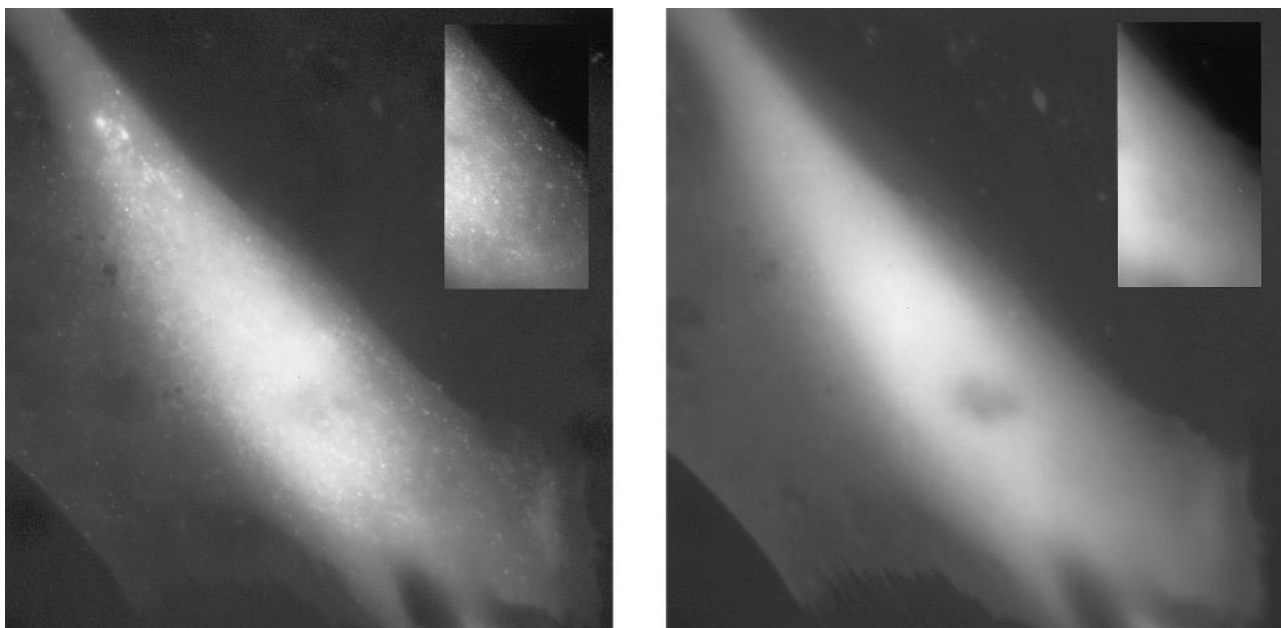
FIG. 1. Imaging of peptide cleavage and trafficking in living cells. This figure illustrates how cleavage indicator peptides can be used to compare the trafficking of cleaved and intact peptide *in vivo.* These two images of the same living cell were taken within 5 s of one another. The cell had been injected in the cytoplasm with a tetramethylrhodamine–Cy5 cleavage indicator. The right-hand image was taken using standard tetramethylrhodamine filters (Chroma Inc, Brattleboro, VT) to visualize the cleaved MHC-I-binding peptide fragment (bearing rhodamine alone) in the ER, while the left-hand image was taken using rhodamine excitation and Cy5 emission wavelengths to visualize the intact peptide, the only species capable of undergoing FRET. Insets show the same regions of each image. The intact peptide has proceeded to the intermediate compartment prior to the MHC-I-binding fragment. Identification of the fluorescent compartments through costaining with antibody markers and other controls is discussed elsewhere.

cients, quantum yields, fluorescence lifetimes, and relative orientation of the two dyes (1). There are many suitable, commercially available dyes that bear reactive groups required for the labeling methods described here (2). We have had success with fluorescein–tetramethylrhodamine and tetramethylrhodamine–Cy5 FRET pairs, but other fluorophores have also been used successfully (2). To avoid perturbation of biological behavior, experiments must be performed using low indicator concentrations. For this reason, it is important to use the brightest and most photostable dyes possible. The choice of dye can of course affect the biological activity of the peptide. Fortunately, a range of commercially available dyes with widely varying shape and charge distributions are suitable for live cell FRET (2). Dyes can be purchased with reactive groups attached at different positions, to alter dye orientation and optimize biological activity or energy transfer.

Recent work has shown that mutants of green fluorescent protein (GFP) with different fluorescence wavelengths can also be used to monitor FRET in living cells (3). This approach shows great promise, as the indicators can be genetically encoded for cell expression. However, the efficiency of energy transfer with current GFP mutants remains substantially lower than that with dyes, both because these mutants do not have optimal excitation and emission spectra and because the proximity of two GFP fluorophores is limited by their position buried in the center of the protein. In our hands, the sensitivity and kinetic resolution of *in vivo* cleavage experiments have required a strong FRET signal, as sometimes small amounts of cleaved material must be detected over strong background fluorescence from large quantities of intact indicator. Thus we have chosen to use cleavage indicators based on dyes.

PRODUCTION OF CLEAVAGE INDICATORS

In the following sections, we discuss two methods for introducing one dye at any precise position within a polypeptide and a second dye at the amino terminus. The first method, solution-phase labeling, is applicable to any peptide from any source. It is the easiest process to use, but is limited to peptides that can be biologically active either without cysteine or without lysine, as one of these side chains must be used as a unique reactive handle for dye attach-

ment. Should both cysteine and lysine be essential to peptide activity, a procedure for producing peptides with several free cysteines and/or lysines using solid-phase labeling is also given. This protocol is more complex and can also produce only peptides labeled at the amino terminus and one internal position. We chose not to include protocols for peptides labeled solely on internal residues, as this is more problematic and requires substantially more expertise in synthesis techniques. Fluorescein–rhodamine labeling is used in the examples given here.

It is important that a fluorescent cleavage indicator is produced in high purity. Large quantities of unlabeled starting peptide and/or free dye must be separated from the desired product. Unlabeled peptide will perturb the biological process under study. Free dye will seriously affect the background fluorescence signal, can prevent accurate assessment of localization because dye is often sequestered in vesicles, and is sometimes toxic in cells. Precise placement of the dyes on the peptide is critical because a mixture of labeled peptides leads to ambiguity in the interpretation of the final results. The cleavage indicator should be tested *in vitro* to show that the relevant biological activity has not been perturbed by the presence of dyes and to characterize the changes in fluorescence on cleavage.

The method of choice for purification of polypeptides is reversed-phase high-pressure liquid chromatography (RP-HPLC; see protocols below), but fastprotein liquid chromatography (FPLC) or ionexchange chromatography can be used for particular peptide sequences (4, 5). Once a pure product has been obtained, it is important to confirm that it has the correct structure. A mass spectrum is very useful, as the labeled peptide will have a unique mass (4). The mass spectrometry (MS) data, together with a UV–Vis absorbance spectrum, will confirm the attachment of both dyes. Depending on the application, it may be sufficient simply to demonstrate that the indicator remains biologically active and that FRET is perturbed on cleavage *in vitro.* If necessary, the precise positions of dye attachment can be confirmed by MS analysis of protease fragments or by MS sequencing.

LABELING APPROACH

Three nucleophilic groups in peptides are far more reactive than all others and so can be selectively

labeled. These are the ϵ-amine of lysine, the amino terminus, and the cysteine sulfhydryl group. Selectivity in labeling can be obtained because the reactivity of these groups can be modulated by controlling the pH of the reaction, and because dyes are commercially available that selectively label amines versus sulfhydryl groups (6). Selective attachment of two dyes to peptides is primarily an issue of using the right reactive dyes in sequence, while manipulating the reaction pH.

The reaction pH is important because it controls the protonation state and, hence, the reactivity of the nucleophilic peptide groups (6). The acidity constants (pK_a) for the reactive functional groups are ~9.5 for the ϵ-amine of lysine, ~7.5 for the amino terminus, and ~7.5 for the cysteine sulfhydryl (10). Thus, at pH 7.5 the ϵ-amine of lysine is predominantly protonated and unreactive, leaving only cysteines and the N-terminal amine as reactive sites. To distinguish between the cysteine and the amino terminus, selective reactive groups incorporated into the dyes are used. Isothiocyanates and N-hydroxysuccinimidyl esters react predominantly with amines, while iodoacetamides and maleimides react with sulfhydryl groups (6). This specificity is an intrinsic property of the reactivities of the nucleophilic side chains and the electrophilic dye functional groups in combination, and is a function of hard–soft acid–base chemistry and other factors (11).

SOLUTION-PHASE LABELING

Labeling peptides in solution is an important method for introducing fluorescent labels into any unprotected polypeptide. It is often the only practical method for labeling small quantities of peptides isolated from natural sources. The requirements for this approach are a free amino terminus and the presence of only one lysine or cysteine, which must be used as a nucleophilic handle for labeling. The usual strategy is to label first either cysteine or lysine and then the amino terminus. This labeling process requires purification and product identification at both the single- and double-labeled peptide stages.

Procedure for Solution-Phase Labeling at Cysteine

Cysteine-containing peptide (0.67 μmol) is dissolved at 2.68 mM concentration in 100 mM phos-

phate buffer at pH 7.0–7.5 (250-μL volume) (6). With rapid mixing 6.7 μmol of fluorescein iodoacetamide dissolved in 250 μL of dimethyl sulfoxide (DMSO) is added in two aliquots of 125 μL each, over 2 min. It is important to maintain high concentrations of reactive dye to enable useful rates for the labeling reaction. DMSO of 50% or higher can be used as a cosolvent to maintain solubility of dye, but some buffers are not soluble at such high concentrations of organic solvent. If buffer solubility becomes a problem, the reaction buffer and organic solvent can be premixed, solid can be removed by centrifugation, and the supernatant can then be used for the labeling reaction and to dissolve the dye. After addition is complete, the reaction should be gently stirred at room temperature in the dark and allowed to proceed for 2 to 4 h. The labeled peptide is separated from unreacted dye using gel filtration on a Sephadex G-10 or G-15 column (1.0 × 8.0-cm column) with elution in 100 μM ammonium bicarbonate, which is a volatile salt and can be removed by lyophilization. The labeled peptide will elute from the column as the first, rapidly eluting yellow band, with unreacted dye remaining behind as a yellow band either travelling more slowly or streaking from the top of the column bed. The crude labeled peptide is further purified by RP-HPLC, either directly or after concentration by lyophilization, using a linear gradient of Solvent B in Solvent A [Solvent B = 90% acetonitrile, 10% water, 0.9% trifluoroacetic acid (TFA); Solvent A = 0.1% TFA in water] (4, 7).

Cysteine-containing peptides sometimes form intermolecular disulfide bonds, preventing reaction with dyes. If necessary, disulfide formation can be minimized by using degassed solvents or reducing agents such as 2-mercaptoethanol and dithiothreitol (DTT). These reducing agents themselves contain sulfhydryls which can react with the dyes. Thus they must be removed prior to reaction through buffer exchange (i.e., dialysis or filtration) using degassed solvents. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) is a notable exception and can be useful as a reducing agent that does not react with cysteine-reactive dyes (2, 14).

Procedure for Solution-Phase Labeling at Lysine

Peptide (0.53 μmol) is dissolved in a clean Eppendorf at 2.1 mM concentration of 100 mM sodium carbonate/bicarbonate buffer at pH 9.5 (250-μL volume). A 10-fold excess of Tetramethylrhodamine dye (5.3 μmol) activated as the N-hydroxysuccinimide

ester (commercially available or made as described below) is dissolved in 250 μL of DMSO (6). The solution of activated dye is added to the labeled peptide solution with rapid mixing as described above for cysteine labeling. After the addition is complete, the reaction is left in the dark at room temperature, with either gentle mixing or inversion by hand every 15 min. After 4 h, the peptide is purified by the same two-step gel filtration/RP-HPLC purification described for the cysteine-labeled peptide.

Procedure for Solution-Phase Labeling at the Amino Terminus

Solution phase labeling at the amino terminus is accomplished in the same manner as lysine labeling described above, with one modification. The reaction buffer must be in the pH range 7.0–7.5. Phosphate buffer between 100 and 200 mM is adequate for most peptides (6).

We recommend use of cysteine rather than lysine as the site of internal labeling whenever possible. Labeling at an internal lysine can generate modest amounts of peptide labeled with a fluorescent dye at the amino terminus, which must be separated from the desired product. Labeling at the amino terminus does not generate an appreciable level of dye attachment to the ϵ-amino group of lysine by virtue of the lower pH under these reaction conditions (6). An excellent example of selectively labeling the amino terminus in the presence of a competing lysine by exploiting this property was reported by Wetzel *et. al.* (12).

SOLID-PHASE LABELING

There are two chemistries used for modern solid-phase peptide synthesis, the *tert*-butyloxycarbonyl (Boc) methodology and the fluorenylmethyloxycarbonyl (Fmoc) methodology (4, 5). While the specific details for these two synthetic processes are quite different, the fundamental concepts are the same. A peptide is synthesized on a solid support, with all nucleophilic amino acid side chains protected to block their reactivity. In the final synthesis step, the peptide is both cleaved from the solid support and the side chain protecting groups are removed. By suitable choice of protecting groups, a single nucleophilic site can be unmasked while the peptide is still

on the solid support, and this site can be selectively reacted (7, 8). The chemistry used for solid-phase labeling is identical to that for solution-phase labeling, except that solvents and concentrations are optimized for resin-bound peptides. This is the method of choice when absolute specificity of labeling is required or when peptides must contain multiple unlabeled lysines or cysteines. Purification is a simple process because labeling reactions are driven to completion by excess dye, and excess reagents can be easily washed from the resin-bound products. Intermediate purification after each labeling step is not required. The following procedures are described for Fmoc solid-phase peptide synthesis, the more prevalent method for small peptide synthesis in commercial and research core facilities (4, 5). Here labeling is best performed first at the amino terminus and then at the internal lysine residue. Because the lysine and the amino terminus are both reactive under the conditions used for labeling on the resin, the internal lysine must be protected to permit selective removal of the amino-terminal Fmoc group. After fluorescent dye conjugation at the amino terminus, this lysine must then be deprotected without cleavage of the peptide from the resin. Lysine protected at the ϵ-amine with Mtt (methyltrityl) meets these requirements and is appropriate for Fmoc-based synthesis procedures [available from NovaBiochem, Fmoc-Lys(Mtt)-OH] (8). It is important to use water-free solvents in the following procedures.

Procedure for Dye Activation

The amount of dye required for these solid-phase labeling procedures can be prohibitively expensive. It is much cheaper to convert the free acid form of reactive dyes to the reactive hydroxysuccinimide ester using the procedure given here: Twenty milligrams (0.174 mmol) of *N*-hydroxysuccinimide and 30.6 mg (0.071 mmol, Molecular Probes, Eugene, OR) of 5 (and 6)-carboxytetramethylrhodamine are weighed into Eppendorf tubes. Three hundred microliters of dry dimethylformamide (DMF) is added to *N*-hydroxysuccinimide and mixed gently until dissolved. This solution is added to the carboxytetramethylrhodamine and mixed gently. The solution should not be pipetted up and down for mixing because sample may be lost. To this mixture 10.85 μL (0.070 mmol) of diisopropylcarbodiimide (DIC) is added, and the activation is allowed to proceed for 2 h in the dark with gentle mixing every 15 min. DIC should be handled with caution because it is highly toxic and a sensitizer. The resulting dark precipi-

tate, the urea of diisopropylcarbodiimide, is normal. When adding this activated dye to the resin in the following procedures, another 100 μL of DMF is added and mixed gently, then both the solution and the precipitates are added. This same procedure can be used for the activation of other dyes containing a carboxylic acid functional group (i.e., 5- or 6-carboxyfluorescein, Molecular Probes). While dyes with multiple carboxylic acids can lead to ambiguity about the point of attachment (with respect to dye), one carboxylate is usually preferred over the others (7, 9, 13).

Procedure for Labeling Resin-Bound Peptides at the Amino Terminus

A two hundred milligrams of peptide on the resin ($\sim$0.05 mmol peptide), deprotected at the amino terminus, is placed in a disposable column (Bio-Rad). The peptide–resin is solvated and swelled by gentle agitation (not mechanical stirring) in DMF for 10 min. The resin is then drained of DMF by allowing the solvent to flow through the resin bed and out of the bottom of the column. The resin is properly drained when flow from the bottom of the column stops. A solution of 30 mg of tetramethylrhodamine N-hydroxysuccinimide ester dissolved in 400 μL of dry DMF (generated as described above) is added dropwise to the top of the deprotected peptide–resin bed. This dye solution will displace the residual DMF within the resin, which should be allowed to flow from the bottom of the column. After the dye is added, the dye-saturated resin is mixed until the deep red color is evenly distributed throughout the resin. The ends of the column are covered with Parafilm to prevent flow out of the column, and the reaction is allowed to proceed in the dark at room temperature. After 4–6 h, the unreacted dye is washed off the resin with continuous addition of large quantities of DMF. Washing is continued until the solvent eluting from the bottom of the column is clear. This washing process is then repeated with dichloromethane (13).

Procedure for Labeling at an Internal Lysine

After labeling of the amino terminus on the resin with tetramethylrhodamine, the ϵ-amine Mtt protecting group is removed by five washes with 2 mL of a deprotection cocktail consisting of 1% trifluoroacetic acid and 3–5% triisopropylsilane in dichloromethane (total of 10 mL of deprotection cocktail)

(8, 13). These are highly corrosive reagents that should be handled with care. The deprotection cocktail is added to the top of the resin slowly and allowed to elute from the bottom of the column (displacing the residual DMF and, with each successive wash, the previously added and spent deprotection cocktail). The total time of deprotection should be >10 min. More cleavage cocktail should be added if the deprotection time proves to be insufficient. After deprotection, the resin is washed using 30–40 mL of DMF added to the top of the resin bed and allowed to flow out of the bottom of the column. This wash cycle is very important! After the deprotection reagents are washed off, the DMF is allowed to flow through the resin bed until all flow stops. At this point, the labeling and washing steps are almost identical to those described above for labeling at the amino terminus. The attachment of fluorescein activated as
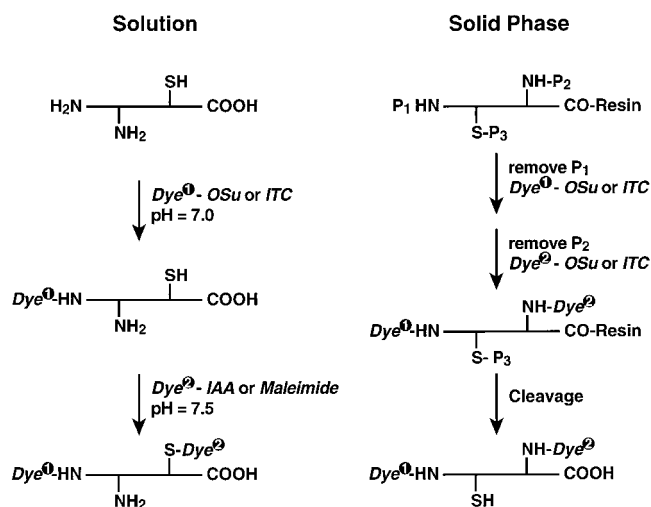
FIG. 2. Strategy for synthesis of cleavage indicators by solution- or solid-phase techniques. *Left:* Solution-phase labeling. An unprotected peptide is first labeled at the amino terminus with Dye 1 activated as the OSu ester or ITC. The internal cysteine of the amino-terminally labeled peptide is reacted at cysteine with Dye 2 activated as the iodoacetamide (IAA) or maleimide to generate the double-labeled peptide cleavage indicator. Alternatively, an internal lysine could be used if unprotected cysteine was required for the function of the peptide. *Right:* Solid-phase labeling. The completely protected synthetic peptide, attached to the resin, is deprotected at the amino terminus by removal of protecting group P_1, then reacted with Dye 1 activated as the OSu ester or ITC. Attachment of the second dye occurs in the same manner: deprotection at lysine by removal of protecting group P_2 followed by reaction with Dye 2 activated as the OSu or ITC. Protecting groups at P_1 and P_2 must be selectively cleavable for this strategy. Cleavage of the labeled peptide from the resin generates the unprotected fluorescent cleavage indicator.

the N-hydroxysuccinimide ester is identical to that used for the coupling of tetramethylrhodamine to the amino terminus. After 4–6 h for dye coupling, the excess activated dye is removed by continuous DMF washing followed by continuous washing with dichloromethane. A final wash cycle with 1:1 methanol:dichloromethane is required to remove all excess unreacted dye. All wash cycles are continued until the solvent eluting from the bottom of the column is clear. The double-labeled peptide–resin is dried *in vacuo* for 16–24 h, then cleaved and purified using the standard protocols performed by the synthesis facility.

CONCLUSION AND SUMMARY

An array of alternate techniques are available for labeling peptides with fluorescent dyes. The procedures described in this article (see Fig. 2) are a limited selection that fulfill two requirements: They can be widely used by biological laboratories with access to commercial or core peptide synthesis facilities, and they are sufficiently versatile to provide access to a wide range of peptide cleavage indicators. We hope these approaches can be applied to defining the biological importance of peptide cleavage events in specific subcellular locations and to examining how these events correlate with the dynamics of downstream effects in real time within living cells.

ACKNOWLEDGMENTS

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