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Multiparameter Flow Cytometric Analysis of a pH Sensitive Formyl Peptide With Application to Receptor Structure and Processing Kinetics[1]

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Environmentally sensitive molecules have many potential cellular applications. We have investigated the utility of a pH sensitive ligand for the formyl peptide receptor, CHO-Met-Leu-Phe-Phe-Lys (SNAFL)-OH (SNAFL-seminaphthofluorescein), because in previous studies (Fay et al.: Biochemistry 30:5066–5075, 1991) protonation has been used to explain the quenching when the fluoresceinated formyl pentapeptide ligand binds to this receptor. Moreover, acidification in intracellular compartments is a general mechanism occurring in cells during processing of ligand-receptor complexes. Because the protonated form of SNAFL is excited at 488 nm with emission at 530 nm and the unprotonated form is excited at 568 nm with emission at 650 nm, the ratio of protonated and unprotonated forms can be examined by multiparameter flow cytometry. We found that the receptor-bound ligand is sensitive to both the extracellular and intracellular pH. There is a small increase in the pK_a of the ligand upon binding to the receptor consistent with protonation in the binding pocket. Once internalized, spectral changes in the probe consistent with acidification and ligand dissociation from the receptor are observed.

Key terms: Multiparameter flow cytometry, ligand-receptor interaction, pH sensitivity, formyl peptide receptor

The human neutrophil formyl peptide receptor is a cell surface receptor linked via a G protein to responses essential in the defense against bacterial infection. The receptor consists of seven α-helical membrane-spanning regions (1), and the binding pocket of such receptors is likely be formed from a cleft surrounded by the membrane spanning regions (18). The neutrophil receptor is rapidly internalized following activation, and its processing (acidification in the lysozomes) and recycling are believed to be required to sustain chemotactic motility over periods of hours (22).

Formyl peptide ligands covalently modified by fluorescent probes have proven to be very useful in the detection and quantification of formyl peptide receptors (12,13). In addition, spectroscopic properties of the bound ligand provide insight into structural features of ligand-receptor interactions on neutrophils (4,14). Experiments employing ligands of different lengths suggested that the binding pocket accommodates no more than six amino acids (14), which is in agreement with earlier studies using nonfluorescent techniques (5,11). The binding of tetrapeptide and pentapeptide fluoresceinated ligands to the formyl peptide receptor is associated with fluorescence quenching. pH-dependent binding studies demonstrated that the binding pocket contains at least two microenvironments: the hydrophobic interior of the pocket contains the tetrapeptide,

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and the exterior region—interacting with the fluorescein of the pentapeptide—is protonating (4). The quenching of fluorescein or derivatized FITC can arise from protonation (6,8) as well as other mechanisms, such as interaction with hydrophobic aromatic residues (e.g., tryptophan) in solution and in protein binding pockets (7,21). Since the response in fluorescence emission in both cases is a decrease in intensity without any spectral variations, one mechanism of quenching is indistinguishable from another based on fluorescence intensity alone. In this paper we therefore took advantage of the environmental sensitivity of the emission spectra of a SNAFL-labeled formyl peptide ligand to investigate the basis of fluorescence quenching upon binding of ligand to the human neutrophil formyl peptide receptor and to confirm previous results concerning the internal environment of the receptor binding pocket. Since ligand bound to receptors could also be protonated during processing by acidic intracellular compartments, changes in the emission of SNAFL also were used to detect the kinetics of environmental pH changes to which the ligand is exposed following receptor internalization.

The results obtained using the SNAFL-labeled ligand support the idea that the formyl peptide receptor binding pocket contains a microenvironment capable of protonating the SNAFL ligand. We also suggest that following internalization, ligand dissociates from the receptor and experiences a time-dependent acidification. These results are consistent with ligand-receptor processing beginning 3–4 minutes after internalization.

MATERIALS AND METHODS

HL60 Cells and Neutrophils

HL60 cells were cultured and differentiated with db-cAMP to express formyl peptide receptor by the method of Chaplinski and Niedel (2). Human neutrophils were prepared by the elutriation method of Tolley et al. (19). Neutrophils were permeabilzed with digitonin as described (16). Briefly, stock solutions of digitonin (1 mg/mL in the intracellular buffer described) were prepared daily. Neutrophils were suspended at 2.5×10^7 per mL in the same buffer and incubated for 25 min at 37°C with 15 μg/mL digitonin. Cells were then washed and resuspended in the appropriate buffer.

Formyl Peptides

The hexapeptide CHO-Nle-Leu-Phe-Nle-Tyr-Lys was obtained from Bachem Bioscience (Torrance, CA). The pentapeptide (CHO-Met-Leu-Phe-Phe-Lys) was prepared by the rapid mixed anhydride procedure (10) as described by Sklar et al. (13) and conjugated with the succinimydyl ester of carboxy SNAFL-2. In the reaction 3 mg of the pentapeptide in 1.0 ml acetonitrite containing 1 equivalent of diisopropylethylamine and 1 equivalent (2.2 mg) of SNAFL-2 succinimydyl ester were reacted at RT for 18 h. The product was purified by HPLC. Peptides were stored as aliquots in DMSO at concentrations ranging from 1 to 10 mM.

Buffer

The binding buffer contained 100 mM KCl, 20 mM NaCl, 1 mM EGTA, and 30 mM Hepes, pH 7.3, and was supplemented with 0.1% BSA, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonylfluoride (PMSF) to stabilize the peptide fluorescence.

Peptide Emission Spectra

SNAFL-pentapeptide was diluted to a final concentration of 1 μM in binding buffer over a pH range from pH 6.5 to 9.0. Fluorescence emission spectra were examined in the photon counting mode in a SPEX spectrofluorometer (Mountain View, CA) outfitted with a quartz cuvette. Excitation was at wavelengths ranging from 488 to 565 nm using a dual monochrometer with emission from 500 to 700 nm through a dual monochrometer.

Spectrofluorometric Assays of Ligand Binding to Cell-Surface Receptors

The spectrofluorometric analysis of ligand binding was based on the method of Sklar et al. (13). Permeabilized neutrophils were suspended at 10^7/mL in binding buffer (pH 7.3) and 200 μL aliquots were placed in a stirred cuvette in the SLM 8000 spectrofluorometer (SLM-Aminco, Champaign-Urbana, IL). The fluorescence was monitored continuously following the addition of fluorescent peptide at a nominal concentration of 5 nM.

Equilibrium Cytometric Binding Assays

The cytometric equilibrium binding assays use a FACScan flow cytometer (Becton Dickinson, San Jose, CA) (3). A suspension of permeabilized neutrophils (10^6/mL) in freshly filtered binding buffer (pH 7.3) was equilibrated in the presence of solutions of SNAFL-pentapeptide (nominal concentrations ranging from 0.01 to 100 nM) for at least 1 h at 4°C. Control ("blocked") samples were made by suspending cells in binding buffer to which had been added excess nonfluorescent blocking peptide, CHO-Nle-Leu-Phe-Nle-Tyr-Lys (10^{-5} M). Histograms of the individual suspensions were acquired at 4°C in the cytometer, and data were expressed as the mean fluorescence channel of the cell suspension. Specific binding was calculated from mean fluorescence channel values of the bound sample minus the mean of the corresponding blocked sample. Equilibrium binding data were analyzed by nonlinear regression using the InPlot computer program (GraphPad, San Diego, CA).

Multiparameter Flow Cytometric Spectral Analysis of the SNAFL-Peptide

Spectral measurements involving cells were made using the multiparameter flow cytometer (MPFC) at

the National Flow Cytometry Resource, at Los Alamos National Laboratory (17). The MPFC has three Coherent lasers (Mountain View, CA) for excitation and can analyze eight parameters simultaneously. For bound ligand, a suspension of differentiated HL60 cells (10^6/mL) in freshly filtered binding buffer (pH 6.5–9.0) was equilibrated with 30 nM SNAFL-pentapeptide in the presence or absence of excess blocking peptide at 4°C, as explained for equilibrium cytometric binding assays. Samples were analyzed flow cytometrically and maintained at 4°C using a waterjacket. For samples containing free ligand, SNAFL-pentapeptide was diluted to a final concentration of 333 nM in binding buffer over the same pH range. Data were collected on the flow cytometer triggered using a pulse generator to trigger data acquisition in the absence of cells (17). Samples were excited first at 488 nm, then at 528 nm, and finally at 568 nm. Stray light was reduced with interference beam splitters, and the emission regions of interest were monitored through interference short-pass/long-pass filter combinations passing bands from 530 to 560 nm, 605 to 615 nm, and 630 to 670 nm, respectively. Specific cellular fluorescence was calculated from the mean fluorescence channel value of each bound sample minus the mean of the appropriate blocked sample at each excitation wavelength. Specific solution fluorescence was calculated from the mean fluorescence channel value of each free fluorescent sample minus the mean obtained using buffers alone. Ratios were calculated from the emission intensity of samples excited at 488 nm divided by the emission intensity resulting from excitation at 568 nm. Data were plotted as a function of pH and normalized so that the limiting values of the ratios of intensities were either one (pH 6.5, protonated) or zero (pH 9.0, unprotonated). Curves were analyzed by nonlinear regression using the InPlot computer program.

Kinetic Cytometric Analysis

Kinetic measurements of the acidification of bound ligand following internalization were performed by first binding intact neutrophils to equilibrium with 30 nM SNAFL-pentapeptide in the presence and absence of excess blocking peptide at 4°C as explained. Samples were then placed on the MPFC and elevated to an internalization permissive temperature using a waterjacket maintained at 37°C. To permit kinetic analysis, samples were excited at 528 nm using an argon-ion laser, and fluorescence emission was monitored through an optical system similar to that described for bound and free ligand spectral measurements. In this case, however, emission at band widths from 550 to 560 nm, 605 to 615 nm, and 630 to 670 nm were monitored simultaneously. Fluorescence and forward light scatter data were acquired vs. time by the repetitive acquisition of sequential histograms at 2 min intervals.

RESULTS AND DISCUSSION

Dependence of SNAFL-Pentapeptide Emission on pH

The effect of pH on the emission spectra of the SNAFL-peptide as shown in Figure 1 was, in general, similar to that for the unconjugated dye (21). As reported by Whitaker et al. (21), SNAFL free dye emission intensity decreases at 550 nm with increasing pH and has an isobestic point at ~625 nm when the excitation wavelength is near 500 nm. As the wavelength of excitation was increased, emission of the protonated form of the dye at 550 nm decreased relative to emission of the unprotonated form of the dye at ~650 nm. For both free dye and SNAFL-peptide, with excitation at 568 nm and emission at 620 nm, the unprotonated form of dye was completely resolved from the protonated form and the emission intensity of the unprotonated form of the free dye or SNAFL-peptide increased monotonically as pH increased. As shown in Figure 1, there are also differences in the behavior of the SNAFL peptide as compared to the free dye below pH 7.5. The emission intensity at 550 nm decreased with pH and lacked a well-defined isobestic point when excited either at 488 or 528 nm. In the unconjugated dye at pH values above neutrality, spectral response is attributed to the protonation state of the benzo[*c*]xanthene hydroxyl group. At lower pH values, the protonation state of the two phenyl carboxyl groups (21) is also believed to contribute to the spectral response. The data in Figure 1 may reflect in the SNAFL-peptide contributions of the phenyl carboxyl groups above pH 6.0.

Binding of SNAFL-Pentapeptide to Cells

Equilibrium binding of SNAFL-peptide to permeabilized neutrophils was performed to verify specific binding of ligand to the formyl peptide receptor on cells. Since the spectral emission profile of protonated SNAFL-peptide at pH 7.3 has substantial intensity over a wavelength range from approximately 500 nm to more than 600 nm (Fig. 1), the fluorescence of cells equilibrated in the presence of SNAFL-peptide was analyzed simultaneously on both FL1 (515–545 nm) and FL2 (563–607 nm) channels of a FACScan flow cytometer. Net cellular fluorescence (not corrected for relative differences in signal intensity) was calculated and plotted as a function of the nominal total peptide concentration, as shown in Figure 2A. Since calibration standards are not available for SNAFL, no attempt was made to measure the absolute concentration of free SNAFL-peptide in the binding assays, nor the absolute quantity of ligand bound to cells, as had been done with fluoresceinated ligands in earlier studies (3). At both wavelengths, SNAFL-pentapeptide demonstrated saturable binding over a nominal ligand concentration range up to 100 nM, with an average approximate dissociation constant calculated from the data of 2.3 nM. This represents a binding affinity within one or two

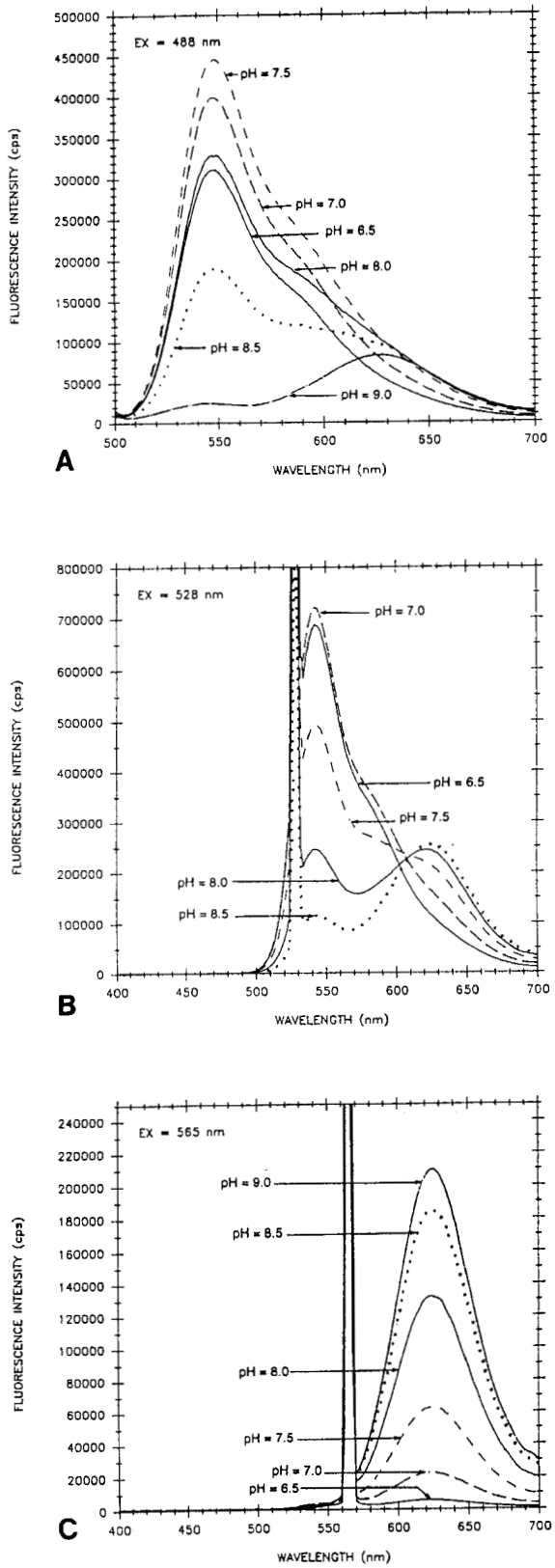

FIG. 1. SNAFL-pentapeptide emission spectra as a function of pH and excitation wavelength. The fluorescent emission spectra of SNAFL-pentapeptide was obtained over the pH range of 6.5–9.0. Excitation was at 488, 528, or 565 nm, as shown in the upper, middle, and lower panels **A**, **B**, and **C**, respectively.

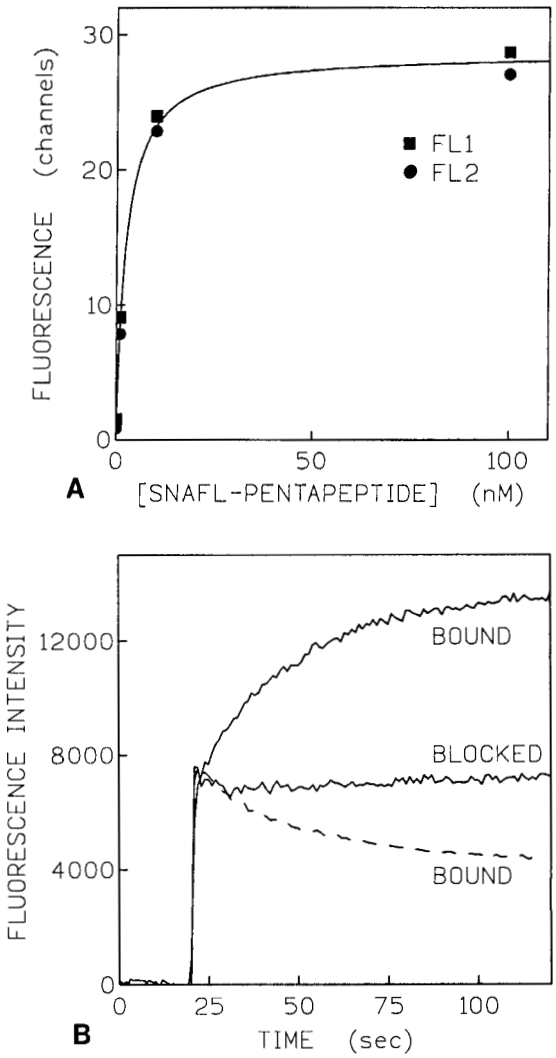

FIG. 2. Binding of SNAFL-pentapeptide to permeabilized neutrophils. **A:** Equilibrium binding. Permeabilized neutrophils were bound to equilibrium with 30 nM SNAFL-pentapeptide in the presence or absence excess nonfluorescent peptide. The bound ligand was analyzed either from FACScan channels FL1 or FL2. **B:** Spectrofluorometric binding of ligand to neutrophils. SNAFL-pentapeptide (solid lines) was reacted with permeabilized neutrophils in the presence or absence of excess blocking peptide and monitored spectrofluorometrically. A similar experiment conducted in the absence of blocking peptide with fluoresceinated pentapeptide (dashed line) from an earlier study is shown for the comparison.

orders of magnitude of the fluoresceinated pentapeptide (3).

Binding is also detected by spectrofluorometry (Fig. 2B) in permeabilized neutrophils in real time using fluoresceinated or SNAFL pentapeptide (3,14). Earlier studies demonstrated that fluoresceinated ligand was quenched upon binding to the formyl peptide receptor, and more recent pH-dependent binding studies (4) suggested that the fluorescein label on the pentapeptide is quenched by protonation. In contrast, the intensity of the SNAFL-pentapeptide increases upon binding to the

cells in response to the environment within the binding pocket. This observed increase in emission intensity upon ligand binding at neutral pH is expected if the probe were well behaved during protonation in solution (21). Anomalies in the behavior between unconjugated and conjugated SNAFL in solution or free and bound conjugated dye may result from differential protonation of the phenyl carboxy groups in the conjugated dye. Thus protection of these groups from protonation within the hydrophobic region of the binding pocket could explain the increase in intensity of SNAFL-pentapeptide upon binding to the receptor.

pH Within the Binding Pocket

The sensitivity of the fluorescence emission of SNAFL to pH was used to assess the environment in the pocket. The ratio of protonated and unprotonated forms of free dye, free peptide, and receptor-bound peptide was examined between pH 6.5 and 9.0 by multiparameter flow cytometry and spectrofluorometry (Fig. 3). The responses of free dye, free SNAFL-peptide, and bound SNAFL-peptide are comparable. As seen in Figure 3 (lower panel) for the SNAFL-peptide, the ratio of the intensity of the protonated form (excitation 488 nm; emission 550 nm) to the unprotonated form (excitation 568 nm; emission 620 nm), whether bound or free, is well behaved and monotonic with pH. As the experimental pH increased, emission from the protonated form decreased, and emission from the unprotonated form increased. The pH values for the half-maximal points were calculated and found to be pH 6.9 for free dye and free peptide, and 7.3 for bound peptide. The small shift is consistent with the idea that protonation occurs within the pocket (4).

Ligand-Receptor Processing

Formyl peptide ligands begin to be internalized approximately 20–30 s after binding and internalization has a half-time of 2–3 min (12). Like other receptors, acidification of the intracellular ligand-receptor complex was suspected. Preliminary data (Sklar, unpublished) suggested that quenching of intracellular fluoresceinated ligand—possibly through acidification)—began approximately 3–4 mins after internalization at 37°C. At 4°C internalization is blocked.

The pH sensitivity of the SNAFL-pentapeptide permits the observation of pH changes associated with intracellular acidification. Figure 4 compares the environment of the probe to morphological changes in the cells evidenced by changes in forward light scatter. These changes in apparent ligand pH and cell morphology ensued rapidly after cells bound ligand at 4°C and were raised to 37°C, a temperature permitting receptor internalization. The changes in the ratio of emission intensities when samples were excited at 528 nm appear to show two phases, an apparent increase in pH within the endosomal vesicle followed by acidification. Since the dissociation half-time of ligand and receptor is in the range of minutes at 37°C, we interpret the

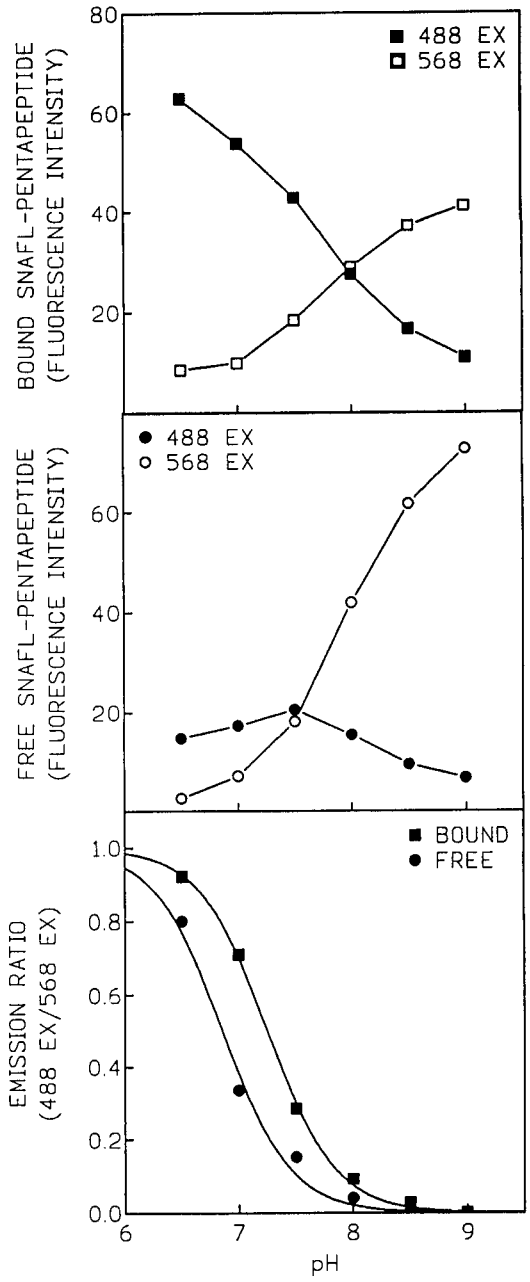

FIG. 3. Multiparameter flow cytometric analysis of free and bound SNAFL-pentapeptide emission spectra. The emission of SNAFL-pentapeptide as a function of pH was analyzed via multiparameter flow cytometry, bound to cells (**top panel**) and free in solution (**middle panel**). Excitation was at 488 nm (closed symbols) or 568 nm (open symbols). The ratios of emission from excitation at these two wavelengths (488/568 nm) was calculated and plotted as a function of pH (**bottom panel**).

decrease in the ratio as dissociation of ligand from receptor at neutral pH, which would have the affect of decreasing the extent of protonation according to Figure 3. Because the ligand may exist in hetergeneous environments (various stages of processing) and be-

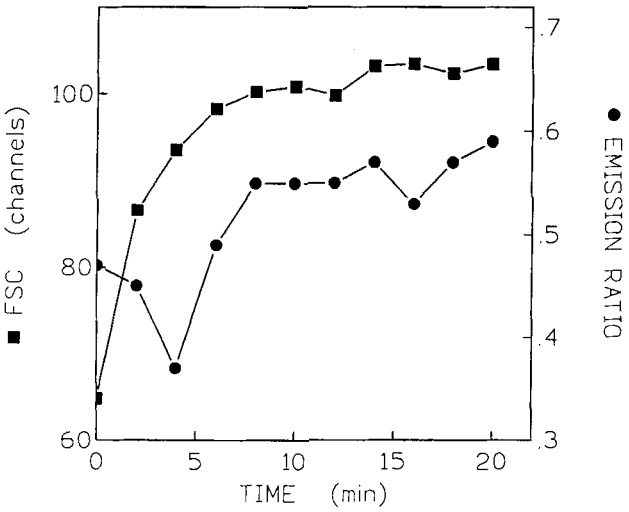

FIG. 4. Multiparameter flow cytometric analysis of SNAFL-pentapeptide processing. Forward light scatter (FSC) and the ratio of emission intensities when samples were excited at either 488 nm or 568 nm (488 nm/568 nm) were plotted as functions of time.

cause the pK_a of the probe is far from the expected internal pH, we cannot accurately quantify the environment in the endosome with this particular ligand.

Implications for Formyl Peptide Receptor Structure and Processing

The use of the SNAFL-pentapeptide in conjunction with multiparameter flow cytometry suggests that probes with well-defined spectroscopy have promise for examining binding pocket structure and ligand-receptor processing. We find evidence for protonation within the binding pocket and following internalization. We have previously suggested that the protonation in the pocket occurs near neutral pH in accordance with the suggestion of Spilberg et al. (15) of a proximal histidine with a pk_a of approximately 7.0–7.5. This study also suggests that upon internalization, ligand dissociates from the formyl peptide receptor and that acidification begins no more than 4 min following internalization. At the time these studies began, the SNAFL derivative was the only pH probe available for conjugation to peptides, even though spectral considerations make it less than ideal for the applications described here. In retrospect it is not surprising that relatively small changes in probe behavior are seen upon binding to receptor, since we now believe that the pk_a of the probe and the pH range for protonation are very similar (4). Moreover, this particular probe is not ideal for detailed analysis of intracellular processing because it is not expected to be very sensitive to anticipated pH ranges of vesicular compartments. This situation is remedied by the newer generation of well-behaved pH sensitive probes, called the NERFS (napthoethylaminorhodofluor) (6).

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