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The use of pepsin in receptor internalization assays

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ABSTRACT

For internalization experiments that use fluorescent antibody (Ab) staining to distinguish between inside versus outside cellular localization of various receptor targeting ligands, it is critical that there be efficient removal of all residual surface-bound fluorescent Ab. To achieve this, a fluorescent Ab removal technique is commonly employed in receptor internalization assays that utilizes low pH glycine-based buffers to wash off the residual non-internalized fluorescent Ab retained on cell surfaces. In this study, we highlight the shortcomings of this technique and propose an alternative *in situ* proteolytic approach that we found to be non-deleterious to the cells and significantly more effective in removing the residual fluorescence resulting from non-internalized surface-bound Ab.

Introduction

Receptor internalization assays are important for understanding how a particular ligand or receptor is involved in endocytosis. What is being measured by these assays is the rate the ligand/receptor pair is internalized into the cytoplasm of the cell. This information can be used for mechanistic studies in multiple areas of research such as receptor-mediated endocytosis and drug uptake.

One common way to explore the rate of cellular uptake for a particular ligand is to label the ligand with a fluorescent tag and estimate the amount of fluorescence that becomes internalized over time by either flow cytometry (FCM) [1] or confocal microscopy [2]. During these ligand/receptor internalization experiments, the ability to distinguish between the amount of ligand that remains on the cell surface and the amount that becomes internalized during the course of the experiment is a significant challenge. With either technique, in order to obtain an accurate estimate of the amount of fluorescence inside the cell that is derived from internalized ligands, it is critical to be able to efficiently wash off 100% of the residual fluorescence (i.e., the fluorescent ligands that were not internalized during the time course of the experiment). If the fluorescent signal remaining on the surface is not close to zero after washing at the end of the internalization experiment, what is measured as the intracellular signal is rendered unreliable [1]. When the ligand analyzed is a targeting antibody (Ab) that has been labeled with a fluorescent tag, removal of the non-internalized Ab from the cell surface can be achieved, but depends on the strength of the washing buffer. When the analyzed ligand is not an Ab but a protein that forms a stable complex with its receptor, removal of residual cell surface fluorescence (i.e., the non-internalized ligands) becomes quite difficult. Nonetheless, when analyzing internalization of a non-antibody ligand that interacts tightly with its receptor, one solution to the residual fluorescence problem would be to label the ligand with a specific fluorescent Ab. The interaction between the fluorescent Ab and the ligand is expected to be weaker than that between the ligand and its cell surface receptor. Therefore, by using buffers that dissociate the Ab from the bound ligand, one should be able to efficiently remove residual fluorescence associated with the ligand. The solution to the residual fluorescence problem in internalization experiments is to find a method that efficiently removes fluorescent Ab from the cell surface.

The classical approach for stripping Ab from cell surfaces requires washing cells at the end of the experiment in a simple acidic buffer containing either 50 mM glycine [1,3,4] or 100 mM Na Acetate [5]. However, after repeatedly testing this approach in our laboratory, we concluded that these buffers are very inefficient at displacing surface-bound Ab. To address this problem, we decided to use a different approach and explore the efficacy of removing surface-bound Ab by employing a proteolytic enzyme known to cleave specific regions of the Ab.

The proteolytic enzyme pepsin, which is crucial for digestive processes in the stomach, is synthesized from pepsinogen and secreted by the gastric chief cells [6]. Pepsin cleaves preferentially at the C-terminal end of aromatic amino acids such as phenylalanine and tyrosine [7]. Pepsin worked most efficiently at removing surface-bound Ab without any detrimental effects on the assay. When incubated with an immunoglobulin G (IgG), pepsin is known to proteolytically separate the Ab into two fragments, the bivalent F(ab′)2 (fragment, antigen-binding) region and the Fc (fragment, crystallizable) region, by specifically cleaving the Ab between these

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two regions [8] (Fig. 1A). We theorized that this proteolytic action of pepsin could also be employed *in situ* to strip away fluorescently labeled Ab from cell surfaces. In our experiments, we used either an anti-integrin or transferrin primary antibody and an Fc-specific Alexa-488 (Invitrogen, Carlsbad, CA) conjugated secondary antibody (Fc A488 secondary Ab) in combination with pepsin digestion to demonstrate, by both FCM and confocal microscopy, that *in situ* proteolysis could effectively remove labeled secondary Ab.

Because pepsin specifically cleaves the Ab at the junction between the F(ab′)2 fragment and the Fc fragment [8], we can take advantage of this cleavage specificity by using an A488 secondary Ab that is Fc-specific. Proteolysis by pepsin will cleave both the primary and the secondary Ab bound on the cell surface. When this happens the F(ab′)2 fragment of the primary antibody will be left in place, but the Fc fragment of the primary Ab along with the bound F(ab′)2 fragments from the secondary Ab will be cleaved off the cellular surface (Fig. 1B). This results in a very efficient removal of any surface fluorescence. If we were to use a secondary Ab that was not Fc-specific, the remaining F(ab′)2 fragment from the primary Ab would still have fluorescent F(ab′)2 fragments from the Fc A488 secondary Ab bound to the cell surface, resulting in persistent signal even after proteolysis.

Materials and methods

Evaluating the efficiency of different stripping buffers on removing cell surface fluorescence. MDA-MB-435 human cancer cells [9,10] were grown in tissue culture flasks and collected by brief trypsinization with 10% trypsin stock (0.05% trypsin–0.02% EDTA) in phosphate buffered saline (PBS) for 5-min. The trypsin was quenched by complete media and the cells were resuspended as needed. We incubated the MDA-MB-435 cells (10^6), in suspension, with a monoclonal Ab against the beta1 integrin subunit (1:500, clone P5D2, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. We then washed the cells three times with PBS and incubated the cells with an anti-mouse Fc A488 secondary Ab (1:1000, Jackson ImmunoResearch, West Grove, PA) for 30 min (the secondary Ab was conjugated to Alexa-488 dye following Invitrogen's protocol). During the labeling process the cells were kept at 4 °C to ensure that there was no membrane traffic and that Fc A488 secondary Ab was not internalized by the MDA-MB-435 cells. These conditions allowed for maximum surface fluorescence to be retained on the plasma membrane (i.e., no membrane traffic) and for the evaluation of the efficiency of different buffers in removing membrane-bound Fc A488 secondary Ab. Three different stripping buffers were analyzed; two were traditional buffers containing either 50 mM glycine, 150 mM NaCl, pH 2.5 (glycine/HCl buffer), or 100 mM Na Acetate, 50 mM NaCl, pH 5.5 (acetate/HCl buffer). For the above buffers the cells were washed for 30 min at 4 °C with gentle agitation. The third buffer was one of the traditional buffers (glycine/HCl buffer) further supplemented with 0.01 mg/ml pepsin (pepsin/HCl buffer). In buffer supplemented with pepsin, cells were washed for 15-min at 4 °C with gentle agitation. After the stripping step, the cells were then either fixed in 3.7% formaldehyde and mounted onto coverslips with fluorescent mounting media (KPL, Gaithersburg Maryland) for confocal microscopy analysis or washed and resuspended in PBS, pH 7.4, for fluorescence-activated cell sorting (FACS) analysis.

Internalization rates of beta1 integrin and transferrin receptors. MDA-MB-435 cells were grown and collected as previously described. Cells (10^6) were labeled with either the beta1-integrin Ab (1:500) or the transferrin receptor (TFR) Ab (1:500, clone 9F81C11, Santa Cruz Biotechnology, Santa Cruz CA), washed and further stained with an anti-mouse Fc A488 secondary Ab (1:1000). Cells were washed three times and incubated with Ab for 30 min at 4 °C. In evaluating beta1 integrin receptor internalization cells were transferred to a 37 °C incubator and internalization of the integrin-bound Ab was allowed to proceed for 5-min, 1-h, or 3-h. Cells with TFR-bound Ab were resuspended in complete media, supplemented with 200 nM transferrin [11], then left to internalize at 37 °C for 5-min, 20-min, or 60-min. At the end of each time point, cells were either washed in glycine/HCl stripping buffer for 30 min, pepsin/HCl-stripping buffer for 15-min, or PBS for 15-min. Washings were done at 4 °C with gentle agitation and the cells were then fixed in 3.7% formaldehyde and mounted onto coverslips with fluorescent mounting media for confocal microscopy.

The confocal images were quantified using the software program Simple PCI (Hamamatsu, Sewickley, PA). Each representative image was scanned, with this software, and every fluorescent pixel was counted that was between the minimum and maximum signal strength which yielded the highest quality image. These same boundaries were applied to all slides. Any pixel above or below the boundary was excluded. The same images were then used to calculate the area of the measured cells. From these two numbers, we obtained the amount of pixels per unit area for each image. This represents the rate that the beta1 integrins or TFRs are internalized per unit area of cell surface.

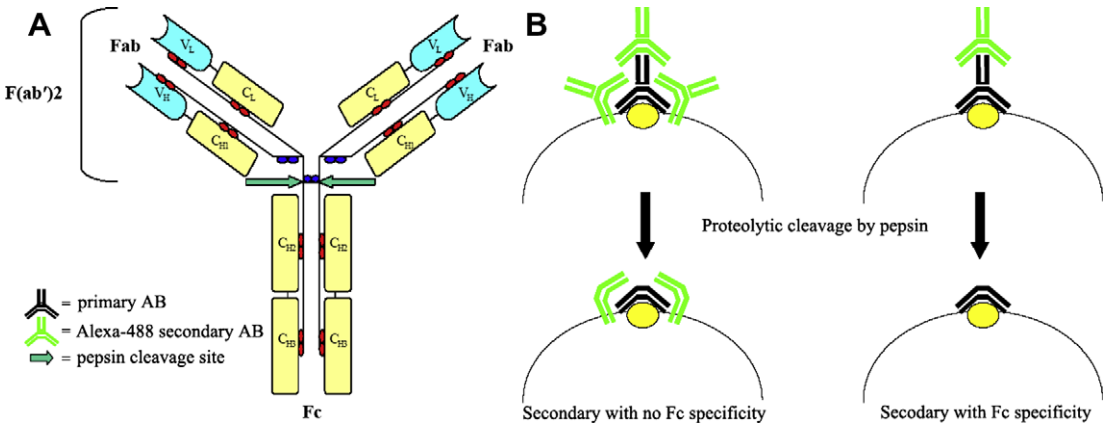

Fig. 1. Fc-specific secondary antibody requirement for pepsin removal of the residual fluorescent signal. (A) Pepsin cleaves antibodies at the junction between the bivalent F(ab′)2 fragment and the Fc fragment. (B) Diagram illustrating the need for an Fc-specific secondary Ab in order for the pepsin cleavage to eliminate the Alexa-488 signal from the cell surface. After protelolytic cleavage, cells that were treated with a non-Fc-specific Ab still have the Alexa-488 F(ab′)2 attached to the primary F(ab′)2, while cells that were treated with the Fc-specific Ab no longer have any portion of the Fc A488 secondary Ab attached to the primary F(ab′)2.