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Direct visualization of protease activity on cells migrating in three-dimensions

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ABSTRACT

Determining the specific role(s) of proteases in cell migration and invasion will require high-resolution imaging of sites of protease activity during live-cell migration through extracellular matrices. We have designed a novel fluorescent biosensor to detect localized extracellular sites of protease activity and to test requirements for matrix metalloprotease (MMP) function as cells migrate and invade three-dimensional collagen matrices. This probe fluoresces after cleavage of a peptide site present in interstitial collagen by a variety of proteases including MMP-2, -9, and -14 (MT1-MMP) without requiring transfection or modification of the cells being characterized. Using matrices derivatized with this biosensor, we show that protease activity is localized at the polarized leading edge of migrating tumor cells rather than further back on the cell body. This protease activity is essential for cell migration in native cross-linked but not pepsin-treated collagen matrices. The new type of high-resolution probe described in this study provides site-specific reporting of protease activity and insights into mechanisms by which cells migrate through extracellular matrices; it also helps to clarify discrepancies between previous studies regarding the contributions of proteases to metastasis.

1. Introduction

Because many proteases exist in both inactive and activated functional forms, clarifying the roles of proteases in cell migration and tumor cell invasion will require examining the localization and focal actions of protease activities rather than the mere presence of protease proteins. For example, even though certain tumor-associated matrix metalloproteases (MMPs) have been linked with cancer metastasis, the significance of these proteases for migration in physiologic tissue environments is controversial; data supporting metastasis as protease-driven, -dependent, -associated, or even irrelevant have been reported (Even-Ram and Yamada, 2005; Lopez-Otin and Matrisian, 2007; Mott and Werb, 2004; Nagase et al., 2006; Overall and Kleifeld, 2006; Sternlicht and Werb, 2001). Even the location of protease activity on the cell surface is uncertain: for example, a study with cells transfected with a FRET-based probe for the individual protease (MT1-MMP) reported localization of MT1-MMP activity at the leading edge of cells migrating on a flat (two-dimensional) substratum (Ouyang et al., 2008). In contrast, in a study of cells migrating in a three-dimensional collagen gel with analysis based on a DQ-collagen probe or antibody detection of a cleaved collagen epitope it was reported that proteolysis occurs much further back on the cell body; it was localized to a collagenolysis zone which was posterior to the leading edge/anterior cell attachment zone of MT1-MMP-transfected HT-1080 cells (Wolf et al., 2007).

Consequently, sensitive high-resolution protease-activity probes are needed to determine the precise localization of proteolysis by living stromal and invading cells, as well as to monitor the capacity of inhibitors to block this local cell-surface activity. This will allow rigorous evaluation of the role(s) and mechanisms of protease involvement in cell migration in three-dimensional settings. Existing probes for whole-cell protease activity (Hobson et al., 2006) and self-quenched probes for protease cleavage (Bremer et al., 2001; Horino et al., 2001; McIntyre et al., 2004; Ouyang et al., 2008; Sloane et al., 2006; Yang et al., 2007) have provided valuable approaches in cell culture and animals. However, an ideal protease probe would be based on a substrate with broad cell-surface protease susceptibility but with high spatial and temporal resolution, and it would be anchored to the extracellular matrix. It should also control for physical environmental effects and should not require transfection or other modifications of the cells being characterized.

We describe a newly designed fluorogenic protease substrate that provides high-resolution, live-cell images of focal protease activity on the surface of individual cells migrating on two-dimensional cell culture surfaces and through three-dimensional collagen matrices. Our goal was to design a probe to detect general matrix-degrading

Abbreviations: MMP, matrix metalloprotease; MT1-MMP, membrane-type matrix metalloprotease-1 (MMP-14); TIMP, tissue inhibitor of metalloprotease; A2, clone of MDA-MD-231 cells expressing MT1-MMP with eGFP marker.

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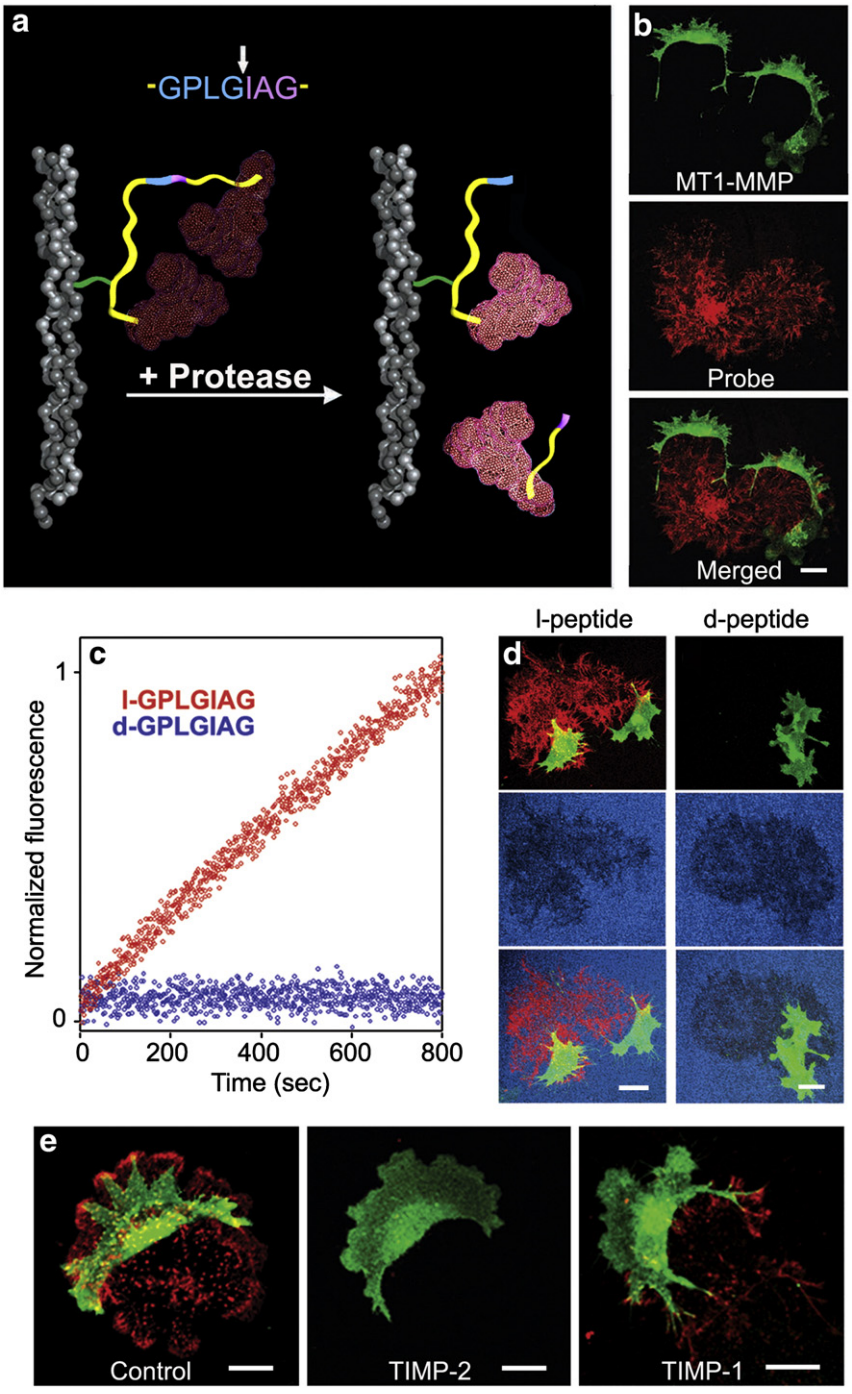

Fig. 1. Fluorogenic probe for detection of protease activity in solution, on a 2-dimensional substratum, or in a 3-dimensional extracellular matrix. (a) The GPLGIAG cleavage site from interstitial collagen is incorporated into a peptidyl backbone (yellow) with constrained conformation (Packard and Komoriya, 2008). The 18 amino acid peptide is then homodoubly labeled with rhodamine 6G fluorophores (red) that self-quench by formation of an intramolecular H-type excitonic dimer (Packard et al., 1996). After cleavage of the GPLGIAG site (between blue and purple in the diagram), two fragments are generated, which separate (one fragment remains immobilized and the other diffuses away), thereby disrupting the intramolecular quenching to generate fluorescence (bright red). The probe can be used to detect protease activities in solution or localized proteolytic activity if immobilized with a biotin-streptavidin linker (green) on a surface such as collagen (gray triple helix). (b) Migrating A2 cells [a clone of breast carcinoma MDA-MD-231 cells expressing MT1-MMP with an enhanced GFP marker] (green) produce fluorescence patterns (red) resulting from cleavage of the probe immobilized on a gelatin substratum. (c) Solution kinetics of cleavage by MT1-MMP show stereospecificity of the probe containing all l-amino acids (red) compared to the control with all d-amino acids (blue). (d) Stereospecificity of l- *versus* d-peptide is retained after immobilization. Cleavage and fluorescence of the probe (red) by A2 cells (green) can be seen in the left column, whereas the right column shows the refractoriness to cleavage of the control (d-amino acids) peptide. Degradation of the gelatin substratum by migrating cells detected by loss of fluorescence (dark areas in blue background) is equivalent. (e) Cleavage of probe (red) by A2 cells (green) on a gelatin substratum is inhibited strongly by 100 nM TIMP-2 compared with untreated control and 100 nM TIMP-1, but migratory morphology is unaffected. Scale bars, 20 μm.

proteolytic activities. Using this novel probe, confocal microscopy, and time-lapse microscopy, we demonstrate localization of protease activity of cells migrating both on 2-dimensional gelatin substrata and in 3-dimensional collagen matrices. Additionally, we show inhibition of cell-surface protease activity and migration by physiologically relevant MMP inhibitors while examining differing conclusions in the literature concerning the cell-surface location of protease activity on cells migrating through three-dimensional extracellular