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Quantitative microtiter fibronectin fibrillogenesis assay: use in high throughput screening for identification of inhibitor compounds

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ABSTRACT

Fibronectin (FN) is a plasma glycoprotein that circulates in the near micromolar concentration range and is deposited along with locally produced FN in the extracellular matrices of many tissues. The control of FN deposition is tightly controlled by cells. Agents that modulate FN assembly may be useful therapeutically in conditions characterized by excessive FN deposition, such as fibrosis, inflammatory diseases, and malignancies. To identify such agents by high throughput screening (HTS), we developed a microtiter assay of FN deposition by human fibroblasts. The assay provides a robust read-out of FN assembly. Alexa 488-FN (A488-FN) was added to cell monolayers, and the total fluorescence intensity of deposited A488-FN was quantified. The fluorescence intensity of deposited A488-FN correlated with the presence of FN fibrils visualized by fluorescence microscopy. The assay Z′ values were 0.67 or 0.54, respectively, when using background values of fluorescence either with no added A488-FN or with A488-FN added together with a known inhibitor of FN deposition. The assay was used to screen libraries comprising 4160 known bioactive compounds. Nine compounds were identified as non- or low-cytotoxic inhibitors of FN assembly. Four (ML-9, HA-100, tyrphostin and imatinib mesylate) are kinase inhibitors, a category of compounds known to inhibit FN assembly; two (piperlongumine and cantharidin) are promoters of cancer cell apoptosis; and three (maprotiline, CGS12066B, and aposcopolamine) are modulators of biogenic amine signaling. The latter six compounds have not been recognized heretofore as affecting FN assembly. The assay is straight-forward, adapts to 96- and 384-well formats, and should be useful for routine measurement of FN deposition and HTS. Screening of more diverse chemical libraries and identification of specific and efficient modulators of FN fibrillogenesis may result in therapeutics to control excessive connective tissue deposition.

1. Introduction

Fibronectin (FN) is a glycoprotein that circulates in soluble form at a concentration range of 200–600 μg/mL (0.4–1.2 μM) in blood and is deposited into detergent-insoluble fibrils in the extracellular matrices in many tissues (Zerlauth and Wolf, 1984; Magnusson and Mosher, 1998). Deposited FN is derived from plasma and also from the cells present in the tissues (Oh et al., 1981; Moretti et al., 2007). Fibrillar FN provides signals for cells to adhere, spread, migrate, proliferate, or differentiate, depending on the context of the microenvironment (Pankov and Yamada, 2002; Mao and Schwarzbauer, 2005). FN fibrillogenesis has been shown to be important for the deposition of other extracellular matrix molecules, including collagen (McDonald et al., 1982; Sottile and Hocking, 2002; Shi et al., 2010), fibrinogen (Pereira et al., 2002), fibrillin (Kinsey et al., 2008; Sabatier et al., 2009), TGF-β binding protein (Dallas et al., 2005), and fibulin (Sasaki et al., 1996), suggesting an orchestrator role for FN in the deposition of connective tissue (Sottile and Hocking, 2002). Thus, FN assembly is fundamental to processes that are restorative, such as wound healing; deleterious, such as malignant growth or fibrosis; or both, such as angiogenesis (To and Midwood, 2011). To enhance or suppress these effects of FN in vivo, one must identify specific modulators of FN fibrillogenesis that can be developed for systemic administration. To this end, there is a need for assays of FN assembly that can be used in high throughput screening (HTS) of small molecule libraries.

Assembly of plasma FN is catalyzed by adherent cells and is dependent on interactions of FN with cell-membrane molecules; these interactions enable the conversion of FN from a compact soluble form to an extended one that forms the detergent-insoluble fibrils (Magnusson and Mosher, 1998; Tomasini-Johansson et al., 2006; Singh et al., 2010). Methods to quantify FN assembly have included the measurement of cell monolayer-bound ^{125}I-labeled FN (McKeown-Longo and Mosher, 1983; Allen-Hoffmann and Mosher, 1987; Tomasini-Johansson et al., 2001) and densitometry of extracted FN detected on Western blots (Wierzbicka-Patynowski et al., 2004; Cho and Mosher, 2006; Xu et al., 2009). These methods are cumbersome, time-consuming, and not scalable. FN assembly can also be assessed by fluorescence microscopy of fluorophore-tagged FN or immunofluorescent detection with anti-FN antibodies (Pankov and Momchilova, 2009). Microscopy offers rich information about fibril appearance, but suffers from field-to-field

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variation and ambiguity about which fields are most representative. Herein we present the development and validation of a robust, straight-forward FN fibrillogenesis assay that can be used in a 96-well plate format for experimental studies or in a 384-well format for HTS. Also presented are the results of a pilot screen of small libraries of compounds with known bioactivity from which a set of compounds has been identified as reproducible, dose-dependent inhibitors of FN assembly.

2. Results and discussion

The assay was designed to allow sequential addition of components to microtiter plate wells with wash steps only at the end. The first step is a 1-h incubation of human skin fibroblasts in 2% fetal bovine serum (FBS) to allow cell adhesion and spreading, which is required for binding and assembly of FN (Zhang et al., 1997). FBS at 2% contains adequate vitronectin to mediate cell adhesion (Hayman et al., 1985). Cell adhesion and spreading in wells were monitored by phase microscopy and observed to be complete 1 h after plating (not shown). The second step is the addition of fluorescently labeled FN, 10–40 nM (4.5–18 μg/mL), in the presence or absence of test compounds. The concentration of FN in FBS is 20–30 μg/mL (Hayman and Ruoslahti, 1979), so the concentration in 2% FBS is <1 μg/mL (<2 nM), considerably less than the concentration of labeled FN. At the end of an incubation period, non-assembled FN is removed by washing, and fluorescence is read on a microtiter plate reader. The number of viable cells remaining in the well is estimated by a luminescent measurement of ATP content using the commercial kit, Cell Titer Glo, thus allowing the amount of assembled FN to be normalized for the number of adherent cells that catalyze assembly and providing a HTS counter-assay for compounds that are cytotoxic or disturb cell adhesion.

The assay was optimized in a 96-well plate format with a flat transparent bottom and black walled wells. We utilized a locally-derived strain of foreskin fibroblasts (AH1F) that synthesize FN, co-assemble endogenous and exogenously added FN, and have been studied previously to identify antibodies that inhibit FN assembly (Peters et al., 1990; Chernousov et al., 1991). We focused on the assembly of fluorescently labeled exogenous FN rather than tagging the assembled total FN at the end of the assay to avoid the additional incubation and wash steps that would be required for the latter. The assay has been tested with embryonic dermal fibroblasts (C1-1-F) and IMR-90 lung fibroblasts obtained from the American Type Culture Collection with similar results (not shown) and should be transferable to almost any fibroblast that adheres to and spreads on microplates in serum-containing medium.

Shown in Fig. 1A is the fluorescence after the addition of 0, 10, 20 or 40 nM A488-FN to 0, 30,000, 60,000, or 120,000 cells per well for 20 h. The signal in wells with no cells or with cells and no A488-FN was approximately 10% of the value with 60,000 cells incubated with 20 nM A488-FN and did not vary when more A488-FN was added in the absence of cells or more cells were added in the absence of A488-FN. These results indicate that the background is not due to the intrinsic fluorescence of cells or non-specific adsorption of A488-FN to wells.

Incorporation of A488-FN measured after 20 h incubation reached plateaus at 40 nM for each cell number, whereas 20 nM was at the cusps of the curves (Fig. 1A); A488-FN at 20 nM (9 μg/mL) was thus chosen as the optimal concentration for the fibrillogenesis assay. We tested whether shorter incubation periods of 1- or 7-h provide a suitable end point, which is reflected by the signal (monolayers with 20 nM A488-FN) to background (monolayers without added label) ratio, S/B. Fig. 1B shows that S/B ratios of approximately 5 can be obtained after a 7-h incubation with 60,000 or 120,000 cells per well, but a higher ratio of about 10 was obtained after a 20-h incubation. Fig. 1C shows that luminescence corresponding to cellular ATP content increases as expected with the cell number per well, with 120,000 cells per well starting to fall off the linear portion of the curve. We therefore chose to use 60,000 cells per well, which made efficient use of cells while preserving signal to background ratio.

The luminescence component of the assay provides a measure of the mass of viable cells after quantitation of fluorescence. As shown in Fig. 1D, 500 nM FUD (Functional Upstream Domain) and 100 μM forskolin, known inhibitors of FN fibrillogenesis (Allen-Hoffmann and Mosher, 1987; Tomasini-Johansson et al., 2001), inhibited normalized (F/L) and raw fluorescence (F) to a similar extent. There was a 15–20% decrease in luminescence promoted by the inhibitors in this context. Importantly, the known inhibitors work by different mechanisms. FUD is a 56-residue polypeptide derived from the F1 adhesin of *Streptococcus pyogenes* that inhibits FN assembly through binding by β-strand addition to multiple N-terminal FN type 1 (F1) modules (^{2}F1–^{9}F1), thus preventing interaction of FN with cell surface molecules involved in its deposition (Tomasini-Johansson et al., 2001; Maurer et al., 2010). Because of its high affinity (nM range) and specificity (Hanski et al., 1996; Ensenberger et al., 2001; Maurer et al., 2010), we used FUD as the preferred prototype inhibitor in further development of the FN assembly assay. Forskolin is a small molecule that acts intracellularly to activate adenylyl cyclase and generate cAMP, causing activation of protein kinase A (Chen et al., 1998).

The 96-well format assay was transferred to a 384-well format with a 4-fold reduction in number of added cells and final volume per well. Addition of cells, library compounds, A488-FN, and washes were performed by robotic systems available at the Small Molecule Screening Facility (SMSF) of the University of Wisconsin Carbone Cancer Center (UWCCC). To corroborate that the HTS assay measuring fluorescence represents fibrillar FN deposition, an inverted fluorescent microscope (BD Pathway) was utilized to image multiple fields in wells of a 384-well plate set up in tandem with fluorescence readings at SMSF. Following washes after 20 h of A488-FN incubation, cell monolayers were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Tween in PBS followed by incubation with rhodamine-phalloidin for 1 h to stain the actin cytoskeleton. As shown in Fig. 2, A488-FN was assembled by AH1F cells into a fibrillar matrix with the expected apical meshwork pattern over cells, which was absent in wells treated with FUD. Each panel is presented as a montage of 4 fields imaged from a given well. Actin stress fibers in the wells treated with FUD appeared similar to those in the A488-FN untreated control. This is consistent with previous results showing that FUD does not visibly affect cell morphology (Tomasini-Johansson et al., 2001; Chiang et al., 2009). Thus, the quantitative microtiter fluorescence assay reflects FN fibrillogenesis.

Shown in Fig. 3A are the averages for positive and negative controls (non-label and FUD) from a 384-well HTS control plate, indicating an S/B ratio of >10. Robustness of a HTS assay is estimated by the Z′ value (Zhang et al., 1999), which is calculated using the formula:

$$Z' = 1 - \left[\left(3sdc^{+} + 3sdc^{-}\right) / \left(mc^{+} - mc^{-}\right)\right]$$

where SD = standard deviation; m = mean; c^{+} = positive control (fluorescent label and no inhibitor); c^{-} = negative control (no fluorescent label or fluorescent label in the presence of a known inhibitor). A Z′ value of 0.4 is considered the minimal robustness for an assay to perform well in HTS (Zhang et al., 1999). We obtained fluorescence Z′ values of 0.67 (n = 80) and of 0.54 (n = 16) for the no label and label along with1 μM FUD, respectively (Fig. 3A). The coefficient of variation was 0.1 (n = 80) for both fluorescence and luminescence assays. When fluorescence was normalized for luminescence, Z′ values were higher, 0.79 and 0.66 for no label and for label along with FUD, respectively. Fig. 3B shows the dose–response curves of F and F/L values obtained with varying concentrations of FUD expressed as the percent of values in wells without FUD. F/L and F were almost identical, indicating little if any effect on cell viability by FUD at any concentration tested. The concentration of FUD that inhibited 50% (IC_{50}) was ~25 nM, which is consistent with values obtained previously in assays of the assembly of ^{125}I-labeled FN (Tomasini-Johansson et al., 2001).