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A non-radioactive dot-blot assay for transglutaminase activity

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Abstract

Aberrant transglutaminase (TG) activity has been implicated in the pathology of numerous diseases including Huntington disease and Alzheimer disease. To fully characterize the role of TGs in these disorders it is important that simple, quantifiable assays be made available. The most commonly used assay currently employed requires significant time and a radioactive substrate. The assay described here uses a biotinylated substrate in conjunction with a dot-blot apparatus to eliminate the use of radioactive substrates, and allows relative transglutaminase activity to be measured simultaneously with minimal sample preparation in a large number of samples containing purified enzyme, cell extracts or tissue homogenates.

Transglutaminases (TGs) belong to a family of enzymes that modify glutamyl residues in protein/peptide substrates usually by transamidation with a suitable amine donor, such as the ϵ -amino group of a lysyl residue or a polyamine. TGs, especially TG2, have been implicated in the pathology of several neurological disorders such as Huntington disease [1,2] and Alzheimer disease [3,4]. TG2 is a highly regulated enzyme that is activated by Ca^{2+} and inhibited by GTP. Currently, the most widely used TG assay utilizes a radiolabeled polyamine (usually putrescine) as acyl acceptor (amine donor) (for a comprehensive review of transglutaminase assays see [5]). This substrate is covalently attached to the glutamyl residue (acyl donor, amine acceptor) of a protein substrate. However, the use of radiolabeled polyamines requires several time-consuming steps, including denaturation of the polyaminated protein with concentrated trichloroacetic acid, followed by filtration, and counting in a liquid scintillation counter. To circumvent the use of radioactivity and problems of waste disposal, 5-(biotinamido)pentylamine has sometimes been used in place of the labeled polyamine as acyl acceptor [6–8].

In the solid-phase system described by Slaughter et al. [8], 0.5 mM 5-(biotinamido)pentylamine was used as acyl acceptor and *N,N*-dimethylcasein coated to polystyrene plates was used as acyl donor. Following incubation with TG, the unreacted 5-(biotinamido)pentylamine was

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removed and the 5-(biotinamido)pentylamine immobilized to the plate was detected with streptavidin-alkaline phosphatase and quantitated by measuring the absorbance increase at 405 nm following addition of *p*-nitrophenol phosphate. The method was used to assay purified guinea pig liver TG and the TG activity associated with plasma Factor XIIIa. The method was also used to detect TG activity in *Escherichia coli* extracts over-expressing Factor XIIIa. The solid-phase system was suggested to be amenable to screening for natural and synthetic inhibitors in extracts of *E. coli* transfected with Factor XIIIa [8]. Although this assay is very sensitive, we envisaged that the procedure could be modified so that the acyl donor *N,N*-dimethylcasein does not have to be coated to wells. This modification would cut down on time and expense. Moreover, in the assay we describe below, we show that it is possible to evaluate the effect of irreversible TG inhibitors in *intact* cells in culture.

Since the 5-(biotinamido)pentylamine is cell permeable it can be added to cells in culture. In theory, this permeability should permit determination of *in situ* TG activity. However, the endogenous TG activity in cells in culture is usually very low [6], presumably as a result, in part, of low intracellular Ca^{2+} levels. To overcome this problem, Zhang et al. [6] used a Ca^{2+} ionophore to increase the intracellular Ca^{2+} concentration in order to activate endogenous TG(s). The cells were lysed, proteins in a portion of the lysate were coated to a well in a 96-well microtiter plate. The supernatant was removed and each well was rinsed, followed by addition of a solution containing horse radish peroxidase (HRP)-streptavidin. After incubation for one hour at room temperature, the wells were rinsed, followed by addition of a solution containing the HRP substrate (*o*-phenylenediamine dihydrochloride). Although capable of measuring TGase activity *in situ* for many cell samples simultaneously, the procedure requires many steps and is time consuming.

Antonyak et al. [7] have used a slightly different strategy. Breast cancer cells were lysed and then exposed to a buffer containing 5-(biotinamido)pentylamine and high Ca^{2+} concentrations. After incubation, the covalently modified proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and then blotted with HRP-streptavidin [7]. The need for a Western blot requires a significant number of steps, is time-consuming, difficult to quantify, and limiting in the number of samples that can be simultaneously analyzed.

The assay described here builds on the previous work of Antonyak et al. [7]. However, the procedure is simplified by using a dot blot apparatus. Moreover, the assay provides an indication of total protein modified rather than a profile of individually modified proteins. Time is saved (SDS gel electrophoresis and transference of protein from the SDS gel to a nitrocellulose membrane are not required) and fewer supplies are needed. As is the case with the procedure described by Zhang et al. [6] and Slaughter et al. [8], the dot blot assay permits a 96-well plate format. However, the present procedure is simpler and more rapid.

Reaction mixtures (50 μl) contained 100 mM Tris-HCl, 20 mM CaCl_2 , 20 mM DTT, 20 μg of *N,N*-dimethyl casein, and 2 mM 5-(biotinamido)pentylamine, pH 7.4. The reaction was stopped by vacuum filtering the reaction mixture through a Bio-dot microfiltration system (Bio-Rad, Hercules, USA) containing a pre-wetted nitrocellulose membrane (Bio-Rad). The membrane was then blocked for 1 hr at room temperature using Odyssey Blocking Buffer (Li-Cor, Lincoln, USA) and incubated with streptavidin labeled with an infrared fluorophore (IRDyeTM CW; $\lambda_{\text{em}} = 774 \text{ nm}$ and $\lambda_{\text{ex}} = 789 \text{ nm}$ (Li-Cor) diluted 1:5000 in blocking buffer (Li-Cor) for 4 hr at room temperature. Biotin incorporation was quantified with an Odyssey imaging system (Li-Cor). Blanks contained EGTA in place of CaCl_2 . [Note that if the Li-Cor system is not available, the nitrocellulose membrane may be blocked with 5% BSA and bound fluorescent proteins visualized with streptavidin-HRP followed by exposure to chemiluminescence reagent and x-ray film, which could then be quantitated using densitometry.]

First we validated the dot-blot procedure with semi-purified guinea pig liver TG2 (>1.5 units/mg; Sigma, St. Louis, USA). TG2 was incubated at 37°C for varying amounts of time. Fig. 1A shows that the rate of covalent attachment of 5-(biotinamido)pentylamine to *N,N*-dimethylcasein is linear for at least 80 min in the presence of 0.5 µg TG2 ($r^2=0.99$). Next, the amount of TG2 added to the reaction mixture was varied and the reaction mixture was incubated for 40 min. Fig. 1B shows that the relative fluorescence intensity increased linearly with increasing amounts of TG2 up to at least 0.8 µg ($r^2 = 0.96$).

Next, we compared the new dot-blot assay to the well established [^{14}C]putrescine-binding assay using guinea pig TG2. The [^{14}C]putrescine-binding assay was as described by Lorand [9]. The reaction mixture (0.5 ml) contained 100 mM Tris-HCl (pH 7.7), 5 mM CaCl_2 , 0.1% lubrol, 5 mM DTT, 0.5 mg *N,N*-dimethylcasein, 0.5 µCi [^{14}C]putrescine (19.0 mCi/ mM; Sigma) and guinea pig liver TG2. After incubation for 40 min at 37°C, the reaction was stopped by transferring the sample to a glass tube containing 5 mL of ice-cold 10% trichloroacetic acid. The label in the denatured protein was then determined as described [8]. Blanks contained EGTA in place of CaCl_2 . The reaction was linear in the presence of 0.5 µg TG2 for about 40 min (Fig. 1C). Fig 1D shows that the amount of label in protein attached to the membrane increases linearly with increasing TG2 up to at least 0.75 µg (40 min incubation).

We then determined whether the dot-blot assay is suitable for measuring TG activity in cell lysates. For this purpose we used immortalized striatal cells derived from a mouse that contained a knock-in of wild type huntingtin expressing a non-pathological polyglutamine region. [This cell model is widely used in HD research.] The cells were lysed by freeze-thawing in lysis buffer (50 mM Tris-HCl (pH 7.4) and 0.1% lubrol) and the cell lysate was incubated at 37°C in a reaction mixture (50 µl) containing 100 mM Tris-HCl, 20 mM CaCl_2 , 20 mM DTT, and 2 mM 5-(biotinamido)pentylamine, pH = 7.5). [Note that addition of *N,N*-dimethyl casein is not necessary when using cell lysates as the 5-(biotinamido)pentylamine is covalently attached to endogenous acyl donor proteins.] Fig 1E shows that the increase in relative fluorescence is linear from 10 min to at least 80 min in the presence of a constant amount of cell lysate (50 µg of protein as measured by the Bradford assay kit (Bio-Rad, Hercules, USA)) ($r^2 = 0.92$). Next, we established that relative fluorescence increases linearly with lysate protein up to at least 200 µg (40 min incubation) (Fig. 1F).

We also validated the new dot-blot assay against the [^{14}C]putrescine-binding assay in the same sample of cell lysate. Cells were harvested, lysed, and a portion of the cell lysate (50 µg of protein) was assayed either with [^{14}C]putrescine or with 5-(biotinamido)pentylamine. The [^{14}C]putrescine-binding assay was carried out as described above except that TG2 was replaced with lysate (50 µg protein). Incubating the reaction mixture with cell lysate at 37°C for 60 min failed to generate a TG activity signal that was significantly above the blank. However, when this amount of lysate was incubated in the presence of 5-(biotinamido)pentylamine for 60 min there was a significant signal above the control (data not show). This finding indicates that not only is the present dot-blot assay more convenient than the [^{14}C]putrescine-binding, it is also more sensitive. TG activity in mouse brain homogenates, as measured by the [^{14}C]putrescine binding-assay, is quite low with a relatively low signal to background ratio [10]. We have found that the 5-(biotinamido)pentylamine dot-blot assay can easily detect TG activity in mouse brain lysates with a signal to background ratio of 3 to 6 (data not shown).

Finally, we investigated the effect of selected TG inhibitors on total TG activity in the striatal cell line by using the dot-blot assay. For this purpose we incubated the cells with 250 µM cystamine (Sigma) or with 50 µM Z-DON-Gln-Ile-Val-OMe (ZDON; Zedira, Darmstadt, Germany; stock solutions dissolved in dimethylsulfoxide) for 12 hr prior to harvest. As seen in Fig 2, when the cell lysate (50 µg of protein) was assayed by the dot-blot procedure (40 min; 37°C), significant loss of activity occurred, especially with ZDON, compared to control cells

treated with vehicle. ZDON is a rationally designed K_{cat} inhibitor of TG2 that covalently adds to the active site cysteine. Cystamine has the potential to inhibit TGs by forming a mixed disulfide with an active site cysteine. However, the cysteine is expected to be regenerated in the assay mixture, which contains a high concentration of DTT. Possibly the mixed disulfide-modified TGase in the intact cells is unstable.

In conclusion, we have described a new TG assay that is rapid, sensitive, has low-signal to noise ratio, is cost effective, does not require radioactive substrates, and can be used in a multi-well plate format with purified enzyme, cells in culture and tissue homogenates.

Abbreviations used

DTT	dithiothreitol
EGTA	ethyleneglycoltetraacetic acid
HD	Huntington disease
HRP	horse radish peroxidase
TG	transglutaminase
TG2	transglutaminase isozyme 2

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Fig. 1. Dot-blot and [¹⁴C]putrescine-binding assays for TG activity. **A)** Dot-blot assay showing linear increase in the fluorescence signal with time in the presence of 0.5 µg semi-purified guinea pig liver TG2. N=3. **B)** Dot-blot assay showing linear increase of fluorescence with concentration of TG2 (40-min incubation time). N=3. **C, D)** As for A and B except that the [¹⁴C]putrescine-binding assay was used. N=3. **E)** Dot-blot assay showing the relationship between relative fluorescence and time in the presence of a striatal cell lysate (50 µg of protein). N=9. **F)** Dot-blot assay showing linear increase of fluorescence with increasing amounts of cell lysate (40-minute incubation time). N= 3; linear regression was used to test for significance; some of the SEM values are smaller than the width of the symbol.

Fig. 2. Effect of pretreatment of striatal cells with two TG inhibitors. TG activity as measured by the dot-blot assay is inhibited by prior treatment of the cells with 250 μ M cystamine or with 50 μ M ZDON relative to control. For details see the text. N = 9, * denotes p value < 0.05; ** denotes p value < 0.01 as measured by Analysis of Variance and Tukey's Multiple Comparison test.