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Distinct Nuclear Localization and Activity of Tissue Transglutaminase*

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Tissue transglutaminase is a calcium-dependent transamidating enzyme that has been postulated to play a role in the pathology of expanded CAG repeat disorders with polyglutamine expansions expressed within the affected proteins. Because intranuclear inclusions have recently been shown to be a common feature of many of these codon reiteration diseases, the nuclear localization and activity of tissue transglutaminase was examined. Subcellular fractionation of human neuroblastoma SH-SY5Y cells demonstrated that 93% of tissue transglutaminase is localized to the cytosol. Of the 7% found in the nucleus, 6% copurified with the chromatin-associated proteins, and the remaining 1% was in the nuclear matrix fraction. *In situ* transglutaminase activity was measured in the cytosolic and nuclear compartments of control cells, as well as cells treated with the calcium-mobilizing agent maitotoxin to increase endogenous tissue transglutaminase activity. These studies revealed that tissue transglutaminase was activated in the nucleus, a finding that was further supported by cytochemical analysis. Immunofluorescence studies revealed that nuclear proteins modified by transglutaminase exhibited a discrete punctate, as well as a diffuse staining pattern. Furthermore, different proteins were modified by transglutaminase in the nucleus compared with the cytosol. The results of these experiments clearly demonstrate localization of tissue transglutaminase in the nucleus that can be activated. These findings may have important implications in the formation of the insoluble nuclear inclusions, which are characteristic of codon reiteration diseases such as Huntington's disease and the spinocerebellar ataxias.

Numerous adult onset neurodegenerative diseases are caused by unstable, expanded CAG trinucleotide repeats within the coding region of an affected gene, which result in the synthesis of disease-specific proteins with expanded polyglutamine domains. Although there are numerous hypotheses concerning the contribution of these proteins with expanded glutamine repeats to the pathogenesis of the diseases, the mechanisms remain unknown (1–4). Recent studies have demonstrated that intranuclear inclusions are a common feature of most of these codon reiteration diseases and that the disease-related protein forms the inclusions (5, 6). However, the underlying mechanisms causing the formation of these inclusions are not clear.

Previously it had been hypothesized that tissue transglutaminase may be involved in the pathological process of the CAG repeat diseases (7). Tissue transglutaminase is both a signal transducing GTP-binding protein (8) and a transamidating enzyme (9). As a member of the transglutaminase family, tissue transglutaminase catalyzes a calcium-dependent acyl transfer reaction between the γ-carboxamide group of a peptide bound glutamine residue and either an ϵ-amino group of peptide bound lysine yielding a isopeptide bond or the primary amino group of a polyamine resulting in a (γ-glutamyl)-polyamine bond (9). Tissue transglutaminase is found within neurons (10, 11) and has been implicated in a variety of processes including apoptosis (12) and axonal growth and regeneration (13, 14). Because the polypeptide bound glutamine is the primary determining factor for a transglutaminase substrate, Green (7) hypothesized that there may be a threshold effect and that the addition of glutamine residues beyond a certain number may allow the mutant protein to be modified by transglutaminase and result in the formation of cross-linked products. Investigators have since demonstrated that peptides containing glutamine repeats are substrates for tissue transglutaminase (15) and that transglutaminase cross-links expanded polyglutamine domains with glyceraldehyde-3-phosphate dehydrogenase resulting in inactivation of the enzyme (16).

In an earlier investigation of GTP-binding proteins in rabbit liver nuclei, tissue transglutaminase was tentatively identified as a nuclear GTP-binding protein (17). However, in this previous study the *in situ* activity of the transglutaminase was not examined. Considering these and other findings, the purpose of this study was to determine the specific nuclear localization of tissue transglutaminase and the *in situ* activation of nuclear tissue transglutaminase.

EXPERIMENTAL PROCEDURES

Cell Culture—Human neuroblastoma SH-SY5Y cells were grown as described previously (18). To induce expression of tissue transglutaminase, cells were grown in the low serum medium containing 20 μM retinoic acid (18). All experiments were carried out 6–9 days after the addition of retinoic acid.

Subcellular Fractionation of SH-SY5Y Cells—Cells were fractionated into cytosolic and nuclear fractions, and the nuclei were further fractionated into Triton X-100-soluble, nuclear chromatin and nuclear matrix compartments as described previously (19). Protein concentrations were determined using the BCA assay (Pierce).

Immunoblotting—To evaluate the expression level of tissue transglutaminase in cell fractions, samples were electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were probed with monoclonal antibody 4C1, which recognizes tissue transglutaminase (20); monoclonal antibody 5H1 (21), which recognizes β-tubulin; or monoclonal antibody MAB052 (Chemicon), which recognizes histone proteins. After incubation with primary antibody, blots were washed and probed with the appropriate horseradish peroxidase-conjugated secondary antibody, washed again, and then developed with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

In Situ Transglutaminase Activity Assay—SH-SY5Y cells were labeled with 2 mM 5-(biotinamido)pentylamine (Pierce), a biotinylated polyamine, for 60 min prior to treatment with 1 nM maitotoxin for 15

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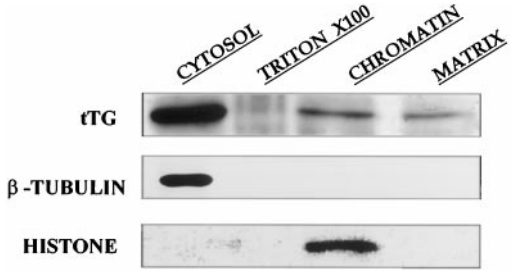

FIG. 1. **Representative immunoblots demonstrating the presence of tissue transglutaminase in cytosolic and nuclear fractions from SH-SY5Y cells.** Cells were separated into cytosol, nuclear Triton X-soluble, nuclear chromatin, and nuclear matrix fractions. Blots were probed for tissue transglutaminase (*tTG*) with the monoclonal antibody 4C1. To verify the extraction and isolation procedure, the fractions were immunoblotted for β-tubulin, a major cytoplasmic protein, using the monoclonal antibody 5H1, and histones, which should localize to the chromatin fraction, using the monoclonal antibody MAB052. Tissue transglutaminase was present in the cytosolic, nuclear chromatin, and nuclear matrix fractions.

min. The cells were subsequently harvested and fractionated into nuclear and cytosolic components (19), and the protein concentrations were determined. Transglutaminase activity was quantitated by measuring the presence of incorporated 5-(biotinamido)pentylamine into proteins by a microplate assay as described by Zhang *et al.* (18). To visualize the proteins into which the 5-(biotinamido)pentylamine had been incorporated, samples (10 μg of protein) were electrophoresed on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated streptavidin (Pierce). The blots were developed as described above.

Immunocytochemistry—SH-SY5Y cells were replated onto poly-D-lysine-coated coverslips. Cells were preincubated for 60 min with 2 mM 5-(biotinamido)pentylamine and subsequently treated with 0.5 nM maitotoxin for 20 min. Tissue transglutaminase was localized with the anti-tissue transglutaminase monoclonal antibody CUB 7402 (NeoMarkers) and Texas Red-conjugated goat anti-mouse IgG (Jackson ImmunoLabs), and transglutaminase activity was detected by FITC-streptavidin[1] staining (18).

RESULTS

Tissue Transglutaminase Co-purifies with the Chromatin and Associated Proteins—To determine the subcellular localization of tissue transglutaminase, cells were separated into cytosolic and nuclear fractions, and then the nuclear fraction was separated further into the Triton X-100 fraction containing lipids and soluble proteins, the chromatin and associated protein fraction, and the nuclear matrix fraction. Equal amounts of protein (10 μg) from each fraction were electrophoresed and immunoblotted with a monoclonal antibody to tissue transglutaminase (Fig. 1). To confirm that the isolated nuclei were free from cytoplasmic contamination, the fractions were immunoblotted for β-tubulin, a predominant cytosolic protein. These results revealed that β-tubulin was found almost exclusively in the cytosolic fraction (Fig. 1). The fractions were also immunoblotted for histones to verify the extraction and isolation of the nuclear fractions. As expected, histone immunoreactivity was present primarily in the chromatin fraction. The presence of histone immunoreactivity in the other fractions was virtually undetectable (Fig. 1). Tissue transglutaminase immunoreactivity was found in the cytosolic, chromatin, and nuclear matrix fractions (Fig. 1). Tissue transglutaminase immunoreactivity in each fraction was determined quantitatively and adjusted for the amount of protein in each fraction. Results from two independent experiments revealed that 93% of total tissue transglutaminase was found in the cytosol, 6% was extracted in the chromatin fraction, and 1% co-purified with the nuclear matrix.

[1] The abbreviation used is: FITC, fluorescein isothiocyanate.

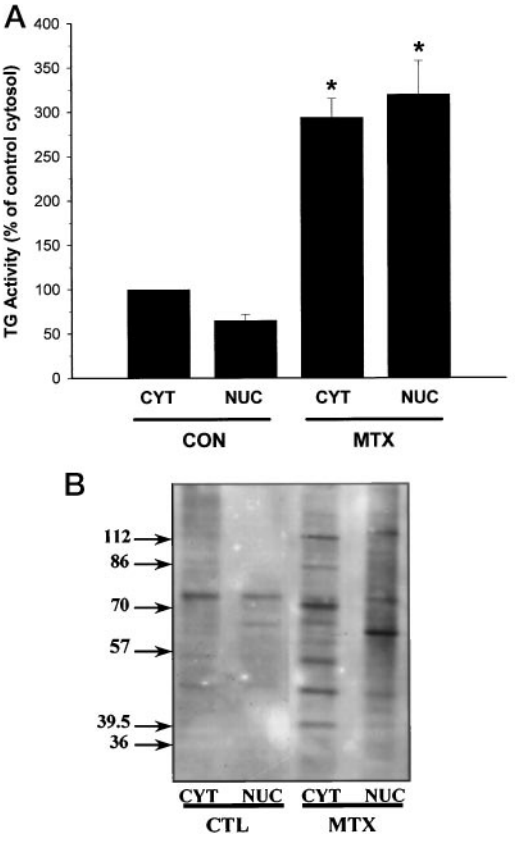

FIG. 2. **Tissue transglutaminase in the nucleus is active.** *A*, quantitative analysis of the effects of increasing intracellular calcium on cytosolic (*CYT*) and nuclear (*NUC*) transglutaminase activity in SH-SY5Y cells. Cells were preincubated with 5-(biotinamido)pentylamine and treated with 1 nM maitotoxin (*MTX*) for 15 min and then separated into cytosolic and nuclear fractions. Both cytosol and nuclear transglutaminase activity were significantly increased by maitotoxin treatment compared with control (*CON*) levels. (*, $p < 0.05$, $n = 5$ separate determinations). *B*, representative blot of the transglutaminase-catalyzed incorporation of 5-(biotinamido)pentylamine into proteins as a measure of *in situ* activity in the cytoplasmic (*CYT*) and nuclear (*NUC*) fractions of control (*CTL*) and maitotoxin (*MTX*)-treated SH-SY5Y cells. Positions at which molecular mass standards (in kDa) migrated are indicated at the *left*.

Nuclear Tissue Transglutaminase Is Active in Situ—To determine if tissue transglutaminase in the nucleus could be activated, an *in situ* transglutaminase assay was used (18). The biotinylated polyamine 5-(biotinamido)pentylamine was used as a probe for endogenous tissue transglutaminase activity. Transglutaminases react with glutamine residues in substrate proteins, and then the enzyme-substrate intermediate reacts with an appropriate nearby primary amine. This can be the primary amino group of a polyamine resulting in the covalent incorporation of the polyamine into the protein by a (γ-glutamyl)polyamine bond (9). Therefore control and treated cells that had been preincubated with 5-(biotinamido)pentylamine were separated into cytosolic and nuclear fractions, and incorporation of this polyamine derivative into proteins was used as a measure of *in situ* transglutaminase activity (22). Fig. 2*A* shows that the basal *in situ* activity of transglutaminase in the nucleus was less than the activity in the cytosol; however, the difference was not significant ($p = 0.056$, $n = 5$ separate determinations). To activate tissue transglutaminase, cells were incubated with 1 nM maitotoxin, which activates both voltage-sensitive and ligand gated calcium channels (23). In SH-SY5Y cells, 1 nM maitotoxin increases intracellular calcium concentrations approximately 10-fold to 700 nM but does not result in loss of viability during the time course of the experiment (18).

In addition, it has been well documented that increasing the cytoplasmic calcium concentration results in an increase in the nuclear calcium concentration (24, 25). Treatment with 1 nM maitotoxin resulted in a significant increase in the *in situ* transglutaminase activity in both the cytosolic and nuclear fractions (Fig. 2*A*). To examine the transglutaminase-induced modifications of nuclear and cytosolic proteins, fractions were blotted with horseradish peroxidase-conjugated streptavidin to identify proteins modified with the biotinylated polyamine. A representative blot of this data is shown in Fig. 2*B*. These findings show that there are different proteins modified by transglutaminase in the nucleus compared with the cytosol.

Cytochemical Analysis of Nuclear Transglutaminase Activity—To further assess the nuclear activity of tissue transglutaminase, a cytochemical approach was used. SH-SY5Y cells were incubated with 5-(biotinamido)pentylamine and then treated with 0.5 nM maitotoxin for 20 min. Control and maitotoxin-treated cells were fixed and immunostained with a monoclonal antibody to tissue transglutaminase and also were probed with FITC-conjugated streptavidin to localize polyamine-modified proteins as a measure of endogenous transglutaminase activity. Transglutaminase was present in control cells (Fig. 3, *A* and *C*), but transglutaminase activity was very low (Fig. 3, *B* and *C*). However, treatment with maitotoxin resulted in a significant increase in transglutaminase activity (as determined by the presence of proteins that had been polyaminated by transglutaminase and visualized with streptavidin-FITC) that was readily evident in the nucleus (Fig. 3, *E* and *F*). In addition, an increased presence of transglutaminase in the nucleus was often observed in the maitotoxin-treated cells (Fig. 3*D*), indicating that transglutaminase may translocate to the nucleus in response to elevated intracellular calcium levels. Tissue transglutaminase and proteins that had been modified by transglutaminase showed nuclear co-localization (Fig. 3*F*). Furthermore, proteins that had been modified by transglutaminase exhibited a discrete punctate as well as a diffuse staining pattern in the nucleus (Fig. 3*E*).

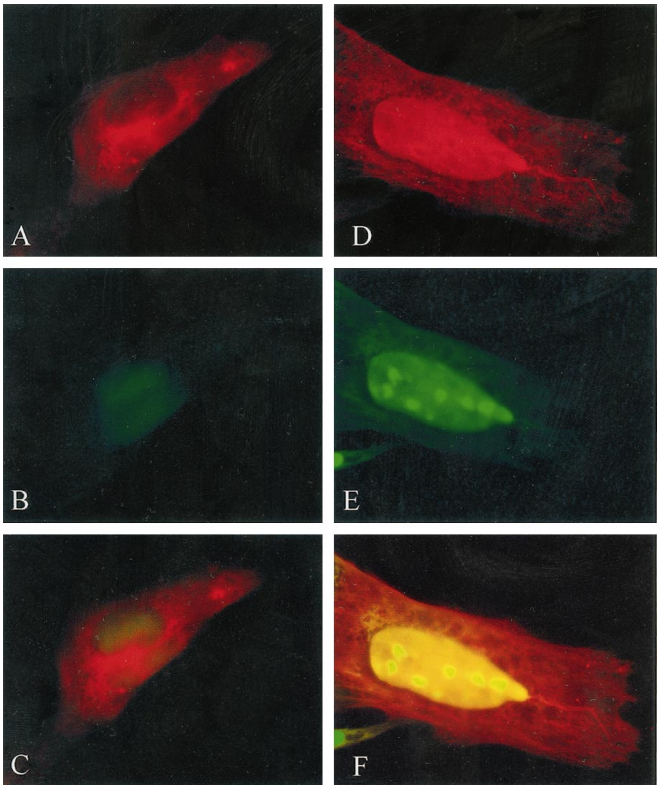

FIG. 3. **Immunocytochemistry analysis of tissue transglutaminase distribution and activity in SH-SY5Y cells.** Cells were labeled with 5-(biotinamido)pentylamine and incubated in the absence or presence of 0.5 nM maitotoxin for 20 min prior to fixation and staining. Tissue transglutaminase localization was detected with the anti-tissue transglutaminase monoclonal antibody CUB 7402 (*A* and *D*), and transglutaminase activity was detected by FITC-streptavidin staining (*B* and *E*). Tissue transglutaminase localization is in *red*, and transglutaminase activity is in *green*. In unstimulated (no maitotoxin) cells (*A–C*), tissue transglutaminase is expressed (*A*), but transglutaminase activity is very low (*B*). In maitotoxin treated cells (*D–F*), tissue transglutaminase expression is evident (*D*), and there is increased staining in the nucleus indicating that tissue transglutaminase may translocate into the nucleus in response to the elevation of intracellular calcium concentration. Significant levels of transglutaminase activity were detected in the nucleus exhibiting both a diffuse, as well as punctate staining pattern of transglutaminase-modified proteins (*E*). Merging of the images demonstrates the colocalization of tissue transglutaminase expression and activity in the cytosol and nucleus of maitotoxin-treated cells (*C* and *F*) (*red + green = yellow/orange*). Original magnification for all images was ×1000.

DISCUSSION

One pathogenic process that has been proposed to contribute to the neurodegeneration in Huntington's disease, as well as other CAG trinucleotide repeat diseases, is the homodimerization or heterodimerization of the mutant polyglutamine-containing proteins with subsequent stabilization by transglutaminase resulting in the formation of poorly soluble protein aggregates (7, 15, 16). This hypothesis has attained additional support with the recent discovery of intranuclear inclusions, which are common to many codon reiteration diseases and are composed of aggregates of the disease-specific protein (5, 6). A previous study presented preliminary evidence that tissue transglutaminase was present in rabbit liver nuclei (17). However, this study focused on the GTP binding properties of tissue transglutaminase and did not examine the *in situ* transamidating activity of the enzyme. Considering the potential pathophysiological importance of nuclear tissue transglutaminase, it was of importance to verify the localization of tissue transglutaminase in the nucleus and to determine whether it had transamidating activity *in situ*. In this study, we clearly demonstrate that tissue transglutaminase localizes to specific nuclear fractions and can be activated *in situ*. Interestingly, when cells were treated with maitotoxin to increase the intracellular calcium concentration, a distinct punctate as well as diffuse pattern of transglutaminase-modified proteins were readily evident in the nucleus (Fig. 3*E*). The identity of the nuclear proteins that have been polyaminated by transglutaminase in the present study are unknown. However, it is intriguing that cotransfection of ataxin-1 containing 30 glutamines and leucine-rich acidic nuclear protein into COS-7 cells resulted in a nuclear distribution similar to that observed for nuclear transglutaminase substrates in this study (26).

The physiological function of nuclear tissue transglutaminase is not known. It has been suggested previously that its G protein activity may be important in the activation of nuclear phospholipase C (17). Interestingly, phospholipase C-δ is found in the nucleus (25, 27), and this is the isoform of phospholipase C, which is likely activated by tissue transglutaminase in its capacity as a signal transducing G protein (28). It has also been demonstrated recently that core histones are excellent substrates of tissue transglutaminase, and the modification of histones by either cross-linking or incorporation of polyamines has been proposed to play both physiological and pathological roles in nuclear function (29). In relation to codon reiteration diseases, it was shown that expanded polyglutamine domains tightly bind to glyceraldehyde-3-phosphate dehydrogenase; however, the enzyme is only inactivated when it is cross-linked to the polyglutamine domain by transglutaminase (16). These findings are extremely intriguing given the recent findings of

Sawa and co-workers (30). They demonstrated that during the cell death process in several cell lines, glyceraldehyde-3-phosphate dehydrogenase is translocated to the nucleus and becomes tightly bound, resisting extraction by DNase or salt treatment. Sawa *et al.* hypothesized that the inability of glyceraldehyde-3-phosphate dehydrogenase to be extracted from the nucleus could be due to covalent cross-linkage to another protein (30). Because transglutaminase localizes to the nucleus and is often up-regulated during apoptosis (31), it may involved in the nuclear modification of glyceraldehyde-3-phosphate dehydrogenase during the cell death process. In addition tissue transglutaminase is also in a position to contribute to the formation of the intranuclear inclusions in the codon reiteration diseases. Further research is required to elucidate the putative roles(s) of nuclear transglutaminase in the cell death process, as well as in the pathology of codon reiteration diseases.

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