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New Concepts

Cancer Isoform of a Tumor-Associated Cell Surface NADH Oxidase (tNOX) Has Properties of a Prion

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ABSTRACT: We have described a drug-responsive form of a cell surface NADH oxidase (hydroquinone oxidase) of cancer cells (tNOX) that exhibits unusual characteristics including resistance to proteases, resistance to cyanogen bromide digestion, and an ability to form amyloid filaments closely resembling those of spongiform encephalopathies and all of which are characteristics of PrP^{sc} (PrP^{res}), the presumed infective and proteinase K resistant particle of the scrapie prion. The tNOX protein from the HeLa cell surface copurified with authentic glyceraldehyde-3-phosphate dehydrogenase (muscle form) (GAPDH). Surprisingly, the tNOX-associated muscle GAPDH also was proteinase K resistant. In this paper, we show that combination of authentic rabbit muscle GAPDH with tNOX renders the GAPDH resistant to proteinase K digestion. This property, that of converting the normal form of a protein into a likeness of itself, is one of the defining characteristics of the group of proteins designated as prions.

Our laboratory has reported a unique cancer-specific (*1*−*3*) and protease-resistant (*4*, *5*) NADH oxidase (tNOX)[1] anchored to the external plasma membrane surface of HeLa cells (*6*), liver tumors of rats (*1*), and human mammary cancer cells (*2*, *3*). It is shed into the sera of cancer patients (*7*, *8*) and into culture media conditioned by the growth of HeLa cells as a 34 kDa peptide (*9*). tNOX is a NADH: hydroquinone oxidase with protein disulfide−thiol interchange activity (*10*, *11*), inhibited by antitumor sulfonylureas (*2*) and vanilloids (e.g., capsaicin) (*3*), that occurs in tissue and sera of patients with both cellular (leukemia and lymphoma) and solid cancers (carcinomas and sarcomas) (*7*, *8*). tNOX activity is absent from the sera of healthy volunteers or patients with diseases other than cancer used as controls. Protease resistance of tNOX has been established for proteinase K, trypsin, subtilisin, V-8 protease, and pronase (*4*, *5*). The protein is also resistant to heat and denaturing agents such as 5 M urea and pH extremes (*5*). If proteinase K digests of the released HeLa protein are subjected to FPLC, the 34 kDa processed (plasma membrane and serum) form of tNOX coisolates in a complex with a 36 kDa peptide identified from the N-terminal amino acid sequence, amino acid composition, and enzymatic activity as muscle glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) (*5*). This mGAPDH resembled mGAPDH isolated from rabbit white muscle in every aspect except for one. The mGAPDH associated with tNOX was resistant both to heat (70 °C) and to proteinase K, whereas its normal cytosolic counterpart, mGAPDH, was neither heat nor proteinase K resistant. This property of proteinase K resistance is used widely to distinguish between the disease (PrP^{sc} or PrP^{res}) and normal PrP^{c} of PrP^{sens}) forms of transmissible spongiform encephalopathy (i.e., scrapie) prions. Should a similar property characterize the tNOX protein, this would represent the first

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[1] Abbreviations: tNOX, tumor-associated plasma NADH oxidase of the outer plasma membrane surface; CNOX, NADH oxidase of the outer plasma membrane surface of normal cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PrP^{sc} or PrP^{res}, disease forms of transmissable spongiform encephalopathy prions; PrP^{c} or PrP^{sens}, normal forms of transmissable spongiform encephalopathy prions; PM, plasma membranes.

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report of prion-like properties for a cancer-related protein. Currently, there is no evidence for a protease-resistant form of mGAPDH in the literature. The hypothesis tested is that mGAPDH of HeLa cells becomes protease resistant through an interaction with tNOX.

Historically, the GAPDH assay has been used as an enzyme to track cellular injury. For example, GAPDH has been used as an indicator of oxidative damage from H_2O_2 on the basis of the ability of H_2O_2 to oxidize the active site cysteine (Cys-149) of mGAPDH (*12*). In this report, GAPDH activity was used as a measure of resistance or susceptibility of the mGAPDH protein to proteinase K.

The source of the GAPDH at the HeLa cell surface is unknown, but GAPDH is concentrated at the inner surface of the plasma membrane where an involvement in endocytosis has been implicated (*13*). One possible source would be from HeLa cells broken during cell culture or harvesting.

Expression of Recombinant Truncated tNOX. A recombinant truncated tNOX was expressed in bacteria and purified. The truncated tNOX protein was comprised of amino acid residues 327−610 of the tNOX protein (284 amino acids). For the expression of the truncated tNOX protein, DNA sequences encoding the corresponding amino acid residues were amplified by PCR using the cloned cDNA of the tNOX carrying a cellulose binding domain as template. *Kpn*I and *Bam*HI restriction sites were incorporated to the PCR-amplified DNA. The PCR product was digested with *Kpn*I and *Bam*HI and ligated to pET-34(b^+) (Novagen, Madison, WI) which was digested with *Kpn*I and *Bam*HI. The resultant plasmid (pET34-ttNOX) was transformed into *Escherichia coli* [BL21(DE3)]. The bacteria carrying the pET34-ttNOX were grown in LB medium containing kanamycin (50 μg/mL) for 16 h at 25 °C.

The cells were harvested by centrifugation (4000*g* for 5 min). The cell pellet was then washed in 20 mM Tris-HCl, pH 8.0, and finally resuspended in 20 mM Tris-HCl, pH 8.0, containing 10 mM DTT and protease inhibitors (1 mM PMSF, 1 mM benzamidine, and 1 mM ϵ-amino-*N*-caproic acid). The cells were then lysed with a French press (20 000 psi, three passages), and the cell lysate was centrifuged at 14 000*g* for 20 min. The supernatant was collected and saved as the source of the solubilized truncated protein (ttNOX-1). The ttNOX-1 was then precipitated by ammonium sulfate, 20% of saturation. Once precipitated, ttNOX-1 was not soluble in 20 mM Tris-HCl, pH 8.0. Refolding of ttNOX-1 was according to the procedure provided by Novagen. Briefly, the pellet was washed with 1% Triton X-100 and ttNOX-1 was solubilized in 50 mM CAPS, pH 11, containing 0.3% *N*-laurylsarcosine sodium salt. Proteins were then dialyzed in two changes of dialysis buffer (20 mM Tris-HCl, pH 8.5, containing 0.5 mM cysteamine and 0.05 mM cystamine) over a period of 14 h, followed by dialysis in two changes of equilibration buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM DTT). After dialysis, ttNOX-1 protein was stored at −20 °C until further use. Expression of ttNOX-1 was analyzed by SDS−PAGE with silver staining and Western blotting. Western blotting was carried out using anti-tNOX monoclonal and polyclonal antibodies. The antibody binding was detected using an alkaline phosphate conjugated to anti-mouse IgG antibody or anti-rabbit IgG antibody.

Assays. Glyceraldehyde-3-phosphate dehydrogenase was isolated from skeletal muscle of New Zealand white rabbits as described by Ferdinand (*14*) or purchased from a commercial supplier (type I from rabbit muscle, Sigma, St. Louis, MO). For assay of enzymatic activity the conditions were 0.1 M Tris-HCl, 0.5 mM EDTA, pH 8.0, 10 mM Na_3AsO_4, 2 mM NAD^+, and 3 mM glyceraldehyde 3-phosphate. NAD^+ reduction was determined from the increase in absorbance at 340 nm at 37 °C measured over 300 s (*14*). A millimolar extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate NADH appearance. To test for protease resistance, GAPDH preparations were mixed with 1 μg/μL, final concentration, fresh proteinase K (Sigma, St. Louis, MO). At various times throughout the digestions (0, 15, 39, 60, 90, and 120 min), aliquots were taken from the reaction mixture and assayed for GAPDH activity over 300 s.

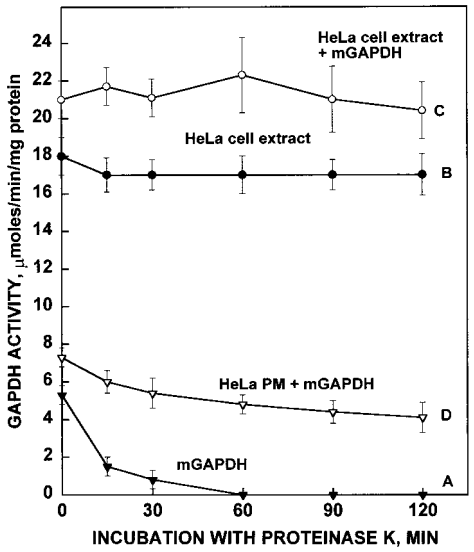

FIGURE 1: Muscle glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) becomes resistant to inactivation by proteinase K in the presence of a low pH extract of HeLa cells enriched in tNOX or with tNOX-containing plasma membranes (PM) isolated from HeLa cells. (A) mGAPDH isolated from rabbit muscle and incubated with proteinase K (1 μg/μL) for 2 h. By 15 min, 90% of the activity was lost and by 60 min, all of the activity was lost. (B) mGAPDH activity of a HeLa cell surface extract containing tNOX treated with proteinase K as for (A). The activity was proteinase K resistant. (C) An equal mixture of mGAPDH (A) and HeLa cell surface extract (B) preincubated for 2 h. The added mGAPDH was now proteinase K resistant. (D) mGAPDH from rabbit muscle made with an equal volume of HeLa cell plasma membranes containing tNOX and preincubated for 2 h. Partial proteinase K resistance was imparted to the GAPDH. The HeLa cell plasma membranes alone contained a GAPDH activity of 2 μmol/min which was proteinase K resistant (not shown). In (A) and (D), protein was based on GAPDH. In (B), the protein amount was based on the HeLa extract. In (C), the protein amount was HeLa extract + GAPDH. Values ± standard or mean average deivations were determined from the time course of GAPDH activity over 300 s from two (plasma membranes) or five (HeLa cell surface tNOX) experiments.

GAPDH Becomes Proteinase K Resistant When Coincubated with tNOX. GAPDH from rabbit muscle (mGAPDH) was susceptible to degradation with proteinase K (Figure 1, line A). However, GAPDH present in extracts of HeLa cells released by treatment for 1 h at 37 °C with 0.1 M sodium acetate, pH 5, was resistant to proteinase K digestion (Figure 1, line B). The N-terminal amino acid sequence of the proteinase K resistant GAPDH present in the HeLa cell extracts was identical to that of the normally proteinase K susceptible form of the mGAPDH. mGAPDH co-incubated

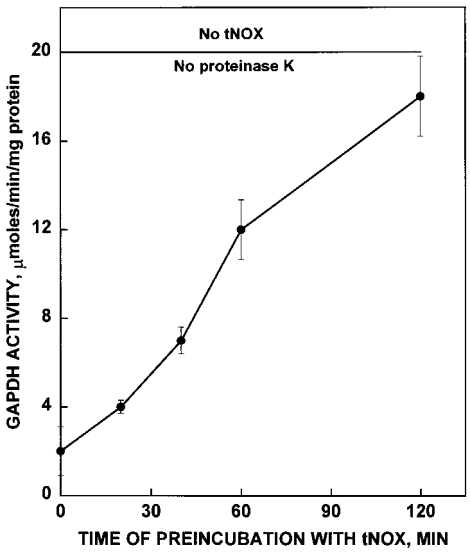

FIGURE 2: Proteinase K resistance is imparted to muscle glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) by preincubation for the times indicated with 0.2 μg of purified, recombinant tNOX, 60 μL total volume. After the preincubation times indicated, portions of 60 μL were removed and treated for 2 h at 37 °C with 10 μg/μL proteinase K. In the absence of preincubation ($t = 0$) the mGAPDH was inactivated. When incubated with recombinant tNOX for the times indicated, the mGADPH gradually acquired proteinase K resistance. mGAPDH not incubated with recombinant tNOX was proteinase K susceptible (no incubation). Control values with no tNOX and no proteinase K were 20 ± 2 μmol/(min·mg of protein). Recombinant tNOX lacks GAPDH activity. Values are from three experiments ± standard deviations.

Table 1: Protection of Commercial Glyceraldehyde-3-dehydrogenase (Type 1 from Rabbit Muscle) by tNOX of HeLa Cell Extract and by Recombinant tNOX

treatment	units/min
no addition	25 ± 3
+proteinase K, 2 h[a]	4 ± 1
+HeLa cell extract, 2 h; proteinase K, 2 h[a]	21 ± 2
+recombinant tNOX, 2 h; proteinase K, 2 h	22 ± 3

[a] Incubation with proteinase K (1 μg/μL final concentration) was at 50 °C, pH 8, for 2 h (ca. 0.1 that of Figure 1). The HeLa cell extract at the dilution used (ca. 0.1 that of Figure 1) contained 2 ± 0.5 units/min GAPDH activity.

with the extracts from the HeLa cell surface at low pH for 2 h at 37 °C also became resistant to proteinase K (Figure 1, line C). In a similar experiment, when mGAPDH was mixed with HeLa cell plasma membrane preparations, the GAPDH activity was again rendered proteinase K resistant (Figure 1, line D). The activity in the absence of proteinase K was stable to incubation of 37 °C.

When incubated in the presence of recombinant tNOX, a similar resistance to proteinase K was imparted to the GAPDH of rabbit muscle (Figure 2). The acquisition of protease resistance occurred more rapidly at 37 °C ($t_{1/2}$ of ca. <30 min) than at room temperature or at 10° C (not shown).

With purified type 1 GAPDH obtained commercially (Sigma), both the HeLa cell extract and the recombinant tNOX resulted in protection of the GAPDH activity from proteinase K inactivation (Table 1). In these experiments and those of Figure 3, the amount of proteinase K was decreased 10-fold, and the temperature of the proteinase K digestion

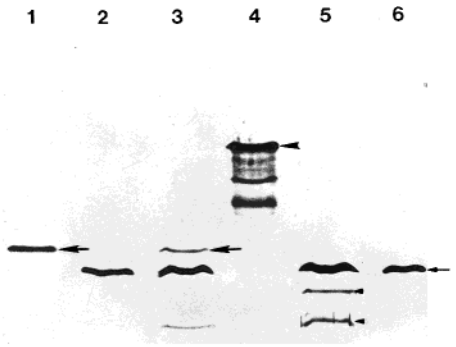

FIGURE 3: SDS−PAGE (10% gel) silver stained to show protection of GADPH with tNOX. Lane 1: Commercial GAPDH (Table 1). Lane 2: Commercial GAPDH + proteinase K (final concentration 1 μg/mL). Lane 3: Commercial GAPDH preincubated for 2 h with recombinant tNOX prior to addition of proteinase K. A significant portion of the GAPDH was protected even with the large excess of proteinase K added and the elevated temperature of incubation. Also remaining was the proteinase K resistant remnant of the tNOX protein (small arrowhead). Lane 4: Recombinant tNOX. Lane 5: Recombinant tNOX + proteinase K (final concentration 1 μg/mL). Proteinase K resistant remnants that retain full tNOX enzymatic activity are indicated by the small arrowheads. Lane 6: Proteinase K (single small arrow) alone. Proteinase K served as a loading control for lanes 2, 3, and 5.

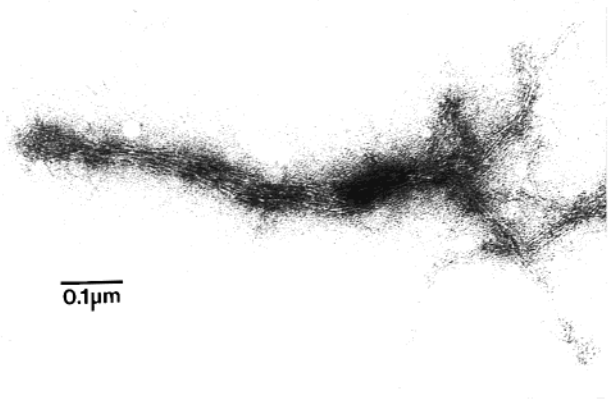

FIGURE 4: Electron microscope picture of a concentrated preparation of purified tNOX treated in the presence of NADH for 30 min. The precipitated proteins were collected and negatively stained with 2% aqueous uranyl acetate. The preparation consisted almost entirely of 10 nm amyloid rods of indeterminate length. Magnification 77500×.

was increased from 37 to 50 °C to enhance reproducibility of the GADPH digestion in the absence of tNOX.

Following assay, the proteinase K digestion was stopped by addition of PMSF, and the resultant fractions were examined by SDS−PAGE (Figure 3). The 37 kDa band of GADPH (Figure 3, lane 1, large arrow) was completely digested by proteinase K (Figure 3, lane 2). However, in the presence of 57 kDa recombinant tNOX carrying the cellulose binding domain (large arrowhead, Figure 3, lane 4), a substantial amount of undegraded GAPDH remained (Figure 3, lane 3). The recombinant tNOX (Figure 3, lane 4, large arrowhead), including several partially processed fragments, was reduced to proteinase K resistant remnants of 29 and 24 kDa (small arrowheads, Figure 3, lanes 3 and 5) which retain NADH oxidase activity (*4*, *5*). Uniformity of loading was indicated by the 33 kDa band of proteinase K (Figure 4, lane 6, small arrow) present in all lanes where added (lanes 2, 3, 5, and 6).

The stabilization of the GAPDH to protease digestion was not due to inactivation of the proteinase K by tNOX or tNOX-containing extracts. Proteinase K, when incubated with tNOX, with or without mGAPDH, digested both serum and egg albumin with equal facility as determined by SDS−PAGE. Complete digestion by proteinase K was found with the above proteins.

The Proteinase K Resistant Form of the Scrapie Prion and tNOX Share Similar Properties. There are a number of similarities among the properties that define both the tNOX protein and the PrP^{sc} prion form of the scrapie protein (*15*). Prions (PrP^{sc}) are regarded as protein forms that multiply by converting a normal form of the protein (PrPc) into a likeness of themselves (*16*). Both preparations of tNOX from the HeLa cell surface and immunoblots of brain homogenates from scrapie-infected hamsters contain proteins with a M_r of 33−35 kDa (PrP $33-35^{sc}$ or tNOX) (*4*, *5*, *15*, *17*). Both PrP^{sc} (*18*) and tNOX (*4*, *5*) are resistant to digestion with proteinase K. Both are plasma membrane located (*6*, *15*, *19*). Both PrP^{sc} and tNOX (*5*) polymerize into insoluble aggregates and/or form characteristic rod-shaped amyloid (Figure 4) (*5*, *20*). Both PrP^{sc} and tNOX polymerize even in the presence of detergents (*21*, *22*). Both PrP^{sc} and tNOX appear to undergo posttranslational modification at the Golgi apparatus. Circular dichroism measurements of the recombinant tNOX protein suggest it to be predominantly β-sheet (65% β-sheet and 8.5% α-helix). This is a further similarity between tNOX and PrP^{sc}. The conformational change that converts PrPc to PrP^{sc} involves largely α-helix to β-sheet transformations (*23*).

The typically felt-like aggregates of ca. 10 nm rigid, linear nonbranching, fibers of indefinite length and with hollow cores on profile are characteristic of all forms of amyloid (*24*). However, a prominent transverse periodicity, seen also with the tNOX fibers at high magnification, is characteristic of the amyloid deposits seen with spongiform encephalopathies.

Despite many functional similarities, the derived amino acid sequence of tNOX (*25*) bears no obvious resemblance to that of the scrapie prion (*15*). The truncated and processed 34 kDa tNOX contains seven cysteines; the scrapie protein has but two (*15*). Also the PrP^{sc} protein is anchored at the cell surface via a phosphatidylinositol glycolipid linkage (*19*, *26*). The tNOX protein lacks such a linkage.

The scrapie prion is a copper binding protein (*27*). In the tNOX protein, a H-V-H motif involving His-546 and His-548 conserved in periplastic copper oxidases together with His-467 forms a potential copper binding ligand (*25*). This H546-V-H-E-F-G motif forms a putative copper binding site that is conserved in both human and chicken superoxide dismutase (*28*). Copper analyses by atomic absorption spectroscopy suggest ca. 1 mol of copper per 34 kDa processed tNOX subunit of the protein purified from sera of cancer patients (*25*). The host-encoded PrPc protein may play a role in normal synaptic function (*29*) and in circadian rhythms (*30*). A function in time keeping (*31*) has been postulated for the constitutive (normal) counterpart of tNOX (designated CNOX) (*32*). While some of the prion−tNOX similarities may be fortuitous, the cancer-related cell surface protein tNOX, which itself is resistant to proteases, does impart protease resistance to GAPDH indicative of a prion-like behavior.

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