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Multiple proteins with single activities or a single protein with multiple activities: The conundrum of cell surface NADH oxidoreductases

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Abstract

Reduction of the cell-impermeable tetrazolium salt WST-1 has been used to characterise two plasma membrane NADH oxidoreductase activities in human cells. The *trans* activity, measured with WST-1 and the intermediate electron acceptor *m*PMS, utilises reducing equivalents from intracellular sources, while the *surface* activity, measured with WST-1 and extracellular NADH, is independent of intracellular metabolism. Whether these two activities involve distinct proteins or are inherent to a single protein is unclear. In this work, we have attempted to address this question by examining the relationship between the *trans* and *surface* WST-1-reducing activities and a third well-characterised family of cell surface oxidases, the ECTO-NOX proteins. Using blue native-polyacrylamide gel electrophoresis, we have identified a complex in the plasma membranes of human 143B osteosarcoma cells responsible for the NADH-dependent reduction of WST-1. The dye-reducing activity of the 300 kDa complex was attributed to a 70 kDa NADH oxidoreductase activity that cross-reacted with antisera against the ECTO-NOX protein CNOX. Differences in enzyme activities and inhibitor profiles between the WST-1-reducing NADH oxidoreductase enzyme in the presence of NADH or *m*PMS and the ECTO-NOX family are reconciled in terms of the different purification methods and assay systems used to study these proteins.

Keywords: CNOX; gp96; NADH oxidase; NADH oxidoreductase; *Trans*-plasma membrane electron transport; WST-1

1. Introduction

The existence of electron transport pathways in cellular compartments other than those of the well-characterised inner mitochondrial and thylakoid membranes has been known for some time. Only recently, however, has the biological significance of these non-mitochondrial pathways been recognised. Of particular interest are the electron transport systems associated with the plasma membrane (reviewed in Ref. [1]). *Trans*-plasma membrane electron transport (tPMET) pathways have been identified in all living cells investigated, and function in biological processes as diverse as cellular defense [2], intracellular redox homeostasis [3], and control of cell growth and survival [4]. Although our understanding of the functional roles of these processes has progressed significantly over the last decade, the molecular identities of the majority of the proteins involved are still unclear. One protein that has received considerable attention, however, is the cell surface NADH oxidase tNOX [5], the gene for which was cloned recently [6].

tNOX is a tumour-associated member of the external NADH oxidase (ECTO-NOX) family of proteins [7].

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; capsaicin, 8-methyl-*N*-vanillyl-*trans*-6-nonenamide; CNOX, constitutive NADH oxidase; ECTO-NOX, external NADH oxidase; EP, pH 5 eluted cell surface proteins; FBS, foetal bovine serum; HBSS, Hanks' Balanced Saline Solution; KCN, potassium cyanide; *m*PMS, 1-methoxy-5-methylphenazinium methylsulfate; NBT, nitro blue tetrazolium; PBS, phosphate buffered saline; pCMBS, *p*-chloromercuriphenylsulfonic acid; PI, preimmune; PM, plasma membrane; PMOX, plasma membrane oxidoreductase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RT, room temperature; SDS-PAGE, SDS polyacrylamide gel electrophoresis; tNOX, tumour NADH oxidase; tPMET, *trans*-plasma membrane electron transport; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt

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Other members of this family include the constitutive NADH oxidase, CNOX [8], and an aging-related protein, arNOX [9]. These proteins exhibit a hydroquinone (NADH) oxidase activity, and a protein disulfide–thiol interchange activity, that cycles every 22 to 26 min [10]. The full-length tNOX gene encodes a protein with a predicted molecular mass of 70.1 kDa [6]. However, the majority of published work has focused on a 34 kDa form of this protein that is shed from the cell surface into culture media [11] or sera [12]. Although the oxidase activity of the ECTO-NOX proteins is usually measured directly by NAD(P)H oxidation, the physiological substrate for these proteins is thought to be plasma membrane hydroquinones [7,13].

To measure a major tPMET pathway in proliferating cells, we have used the cell-impermeable tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium), in conjunction with the intermediate electron acceptor *m*PMS (1-methoxy-5-methylphenazinium methylsulfate) [14]. Whole cell reduction of WST-1 under these conditions is sensitive to inhibitors of glycolysis, stimulated by mitochondrial poisons such as potassium cyanide (KCN), and upregulated in mitochondrial gene knockout (ρ^0) cells that are dependent on glycolytic ATP for survival [15,16]. The substitution of *m*PMS for extracellular NADH provides a second (albeit non-physiological) measure of plasma membrane electron transport, with somewhat different characteristics to that described above [17]. In this latter pathway, the reduction of WST-1 is dependent on added cofactor (NADH or NADPH), but not on intracellular metabolism [17]. The inhibitor profiles of the NADH- and *m*PMS-dependent WST-1-reducing activities differ both from each other and from those of the ECTO-NOX proteins [15]. This is particularly true for the quinone analogue capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide), which stimulates WST-1 reduction in the presence of NADH [17], inhibits *m*PMS/WST-1 reduction [15], and inhibits tNOX [5], but has no effect on CNOX [8]. Although the simplest interpretation of these results is that the different assay systems measure different oxidase proteins, other explanations are possible, and the absence of molecular data for the WST-1-reducing protein(s) makes it difficult to draw definitive conclusions.

To address this issue directly, we set out to identify the cell surface WST-1-reducing NADH oxidoreductase protein from human 143B osteosarcoma cells. This particular cell line was chosen for study because of its interesting cofactor specificity (a preference for NADH over NADPH) with respect to WST-1 reduction [17]. Using blue native polyacrylamide gel electrophoresis, we identified an approximately 300 kDa complex from purified 143B plasma membranes that reduced WST-1 in the presence of NADH. The WST-1-reducing NADH oxidoreductase activity in this complex was attributed to a 70 kDa protein that cross-reacted with antisera against CNOX. In support of the involvement of ECTO-NOX proteins in cell surface WST-1 reduction, we show that previously reported differences in enzyme activities and inhibitor profiles between CNOX and the WST-1-reducing enzyme can be explained in terms of the different purification methods and assay systems used to study these proteins. Taken together, these results suggest that the CNOX and WST-1-reducing NADH oxidoreductase activities are intrinsic to a single multifunctional cell surface protein.

2. Materials and methods

2.1. Materials

The anti-CNOX antiserum [8] was kindly provided by Dr. James Morré (Purdue University, West Lafayette, IN, USA), and anti-gp96 antiserum [18] by Dr. Christopher Nicchitta (Duke University Medical Center, Durham, NC, USA). Human 143B osteosarcoma ρ^0 and parental cells [19] were obtained from Dr. Michael Murphy (Medical Research Council Dunn Human Nutrition Unit, Cambridge, UK). WST-1 and *m*PMS were purchased from Dojindo Laboratories (Kumamoto, Japan). Foetal bovine serum (FBS), GlutaMAX-1, penicillin-streptomycin, and RPMI-1640 medium were from GibcoBRL Life Technologies (Rockville, MD, USA). TEMED, 30% (w/v) acrylamide/bis solution (37.5:1), ammonium persulfate, nitrocellulose and 1 mL Econo-Pac® High Q columns were from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Corporation (East Hills, NY, USA), and Coomassie G250 (SERVA Blue G) from SERVA Electrophoresis GmbH (Heidelberg, Germany). Peroxidase-conjugated goat anti-rabbit immunoglobulins were purchased from DAKO (Glostrup, Denmark), SuperSignal® chemiluminescent substrate from Pierce (Rockford, IL, USA), and 0.5 mL Vivaspin concentrators (5000 MWCO) from Vivascience (Hanover, Germany). All other reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Cell culture conditions

Cells were cultured in RPMI-1640 medium supplemented with 5% (v/v) heat-inactivated FBS, 2 mM GlutaMAX-1, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 50 μg/mL uridine and 1 mM pyruvate, at 37 °C, in a humidified atmosphere with 5% CO_2.

2.3. Preparation of whole cell protein lysates

Cells were washed twice in phosphate buffered saline pH 7.4 (PBS), and resuspended in a lysis buffer containing 50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet-P40, 0.9% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF), at a final concen-