Functional Characterisation of the Ubiquitin-Protein Ligase, Nedd4.

by

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Abstract

Ubiquitination of proteins is a mechanism used by the cell to regulate the function and abundance of a variety of proteins involved in many different cellular processes, including the cell cycle, transcription, signalling and ion transport. Ubiquitin is attached to protein substrates by the coordinated action of three classes of enzymes; ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin protein ligases (E3). E3s are largely responsible for substrate recognition in the ubiquitin pathway. A major subfamily of E3s are the hect proteins which share a conserved domain required for interaction with E2s and an absolutely conserved Cys residue which binds ubiquitin. Among the hect E3s, a number share similar protein domain structure in that they contain an amino terminal C2 domain, 2 to 4 WW domains and a hect domain at the carboxyl terminus. These proteins are known as the Nedd4 family of proteins, as they share homology with the first identified member of this family, Nedd4. The function of these proteins is poorly understood but their unique domain structure suggests that they target membrane proteins for ubiquitin-mediated turnover. The focus of this study was to determine the cellular function of Nedd4.

Caspase-mediated cleavage of proteins during programmed cell death is central to the execution of this cellular process. Only a select group of proteins appear to be cleaved by caspases during programmed cell death. Therefore it is important to identify all caspase substrates so that a clearer understanding of the significance of caspase-mediated proteolysis during cell death can be achieved. Several potential caspase cleavage sites were identified in the Nedd4 protein and therefore its behaviour in apoptotic cells was studied. Nedd4 was shown to be cleaved into two major fragments in a variety of cell lines and in response to a number of apoptotic stimuli. Cleavage of Nedd4 by caspases during apoptosis is likely to be an energy conserving mechanism.

The first protein to be implicated as a ubiquitination target of Nedd4 was the epithelial Na^+ channel (ENaC). ENaC has three subunit types (α, β and γ), each of which possesses a PY motif in the intracellular carboxyl terminus that is capable of interaction with the WW domains of Nedd4. To determine whether Nedd4 mediates ubiquitin-dependent downregulation of ENaC, dominant negative Nedd4 and ubiquitin molecules were tested for their ability to interrupt Na^+-dependent feedback of ENaC in mouse mandibular duct cells. Nedd4 was found to mediate Na^+-dependent feedback of ENaC in a ubiquitin-dependent manner. Additionally it was shown that Nedd4 mediates regulation of ENaC downstream of the G protein, G_o. These findings are of particular importance as Nedd4-dependent regulation of ENaC is likely to be disrupted in the familial hypertensive disorder, Liddle's Syndrome.

ENaC assembles in the membrane as a multimeric complex consisting of 4 to 8 subunits (and therefore contains 4 to 8 PY motifs), while murine Nedd4 contains three WW domains. The requirement of specific Nedd4 WW domains for interaction with ENaC complexes *in vivo* was unknown however. To investigate Nedd4/ENaC interaction, individual ENaC subunits and Nedd4 WW domains were examined for their ability to interact with each other *in vitro* and to interrupt Na^+-dependent feedback of ENaC in mouse mandibular duct cells. All 3 WW domains of murine Nedd4 were shown to be required for regulation of ENaC, although only WW domains 2 and 3 physically interacted with ENaC *in vitro*. This suggested that another, as yet unidentified protein, is involved in ENaC regulation that binds to WW domain 1 of Nedd4 and serves to recruit Nedd4 to ENaC and/or stabilise the Nedd4/ENaC complex.

To determine which ENaC subunits were involved in the regulation of activity of this channel, the ability of the PY motif-containing regions of α, β and γ ENaC to inhibit Na^+-dependent regulation of ENaC was tested. Overexpression of β and γ ENaC, but not α ENaC

was shown to inhibit the Na^+- feedback pathway in mouse mandibular duct cells. This suggests that when cytoplasmic Na^+ levels are increased, the PY motifs of β and γ ENaC, but not α ENaC are required to bind to a negative regulatory protein, presumably Nedd4. The carboxyl termini of β and γ ENaC were also shown to have an important role in positive regulation of ENaC function when Na^+ import is required by the cell. The carboxyl termini of β and γ ENaC therefore appear to be important sites for interaction with protein(s) that both positively and negatively influence channel activity.