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Transduction of NeuroD2 protein induced neural cell differentiation

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Abstract

NeuroD2, one of the neurospecific basic helix–loop–helix transcription factors, has the ability to induce neural differentiation in undifferentiated cells. In this paper, we show that transduction of NeuroD2 protein induced mouse neuroblastoma cell line N1E-115 into neural differentiation. NeuroD2 has two basic-rich domains, one is nuclear localization signal (NLS) and the other is basic region of basic helix–loop–helix (basic). We constructed some mutants of NeuroD2, $ND2_{\Delta 100–115}$ (lack of NLS), $ND2_{\Delta 123–134}$ (lack of basic) and $ND2_{\Delta 100–134}$ (lack of both NLS and basic) for transduction experiments. Using these proteins, we have shown that NLS region of NeuroD2 plays a role of protein transduction. Continuous addition of NeuroD2 protein resulted in N1E-115 cells adopting neural morphology after 4 days and Tau mRNA expression was increased. These results suggest that neural differentiation can be induced by direct addition of NeuroD2 protein.

Keywords: Neural differentiation; Protein transduction; NeuroD2; N1E-115; Basic helix–loop–helix transcription factor

1. Introduction

Regulation of cell differentiation is an important technique in cellular engineering and numerous techniques related to cell differentiation have been developed. Of these, the use of tissue specific transcription factors has received considerable attention.

Cellular differentiation is regulated by tissue specific transcription factors. The neurospecific transcription factor NeuroD2 is highly homologous to other neurospecific transcription factors such as NeuroD and Neurogenin. These transcription factors have a basic helix–loop–helix (bHLH) structure that is character-

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ized by the presence of a basic region adjacent to an amphipathic helix, a loop and a second amphipathic helix that plays an important role in neural development (Lee, 1997; Massari, 2000). Lee et al. (1995) reported that forced expression of NeuroD led to the formation of neurons and Farah et al. (2000) reported that transient expression of NeuroD2 had the ability to differentiate pluripotent mouse P19 embryonal carcinoma cells into neurons. Similarly, Mie et al. (2003) found that the conditional expression of NeuroD2 was capable of inducing neural differentiation. These findings suggest that neural cell differentiation may be regulated by exogenous expression of bHLH transcription factors.

In these experiments, gene transfection was applied using conventional techniques to express transcription factors. However, such methods are often associated with problems such as cytotoxicity, high immunogenicity and genome integration. We therefore employed protein transduction methods to minimize such risks. The methods used to affect protein transduction involved the addition of purified protein to the exogenous environment of cells. Most proteins cannot be transduced into cells by themselves and usually require being fused to short peptides, such as Protein Transduction Domain (PTD) or Cell Penetrating Peptide (CPP), that are capable of penetrating the cellular membrane. Studies have demonstrated that various PTD-fused proteins can be transduced into cells and that they are capable of regulating cell functions (Schwarze et al., 1999; Phelan et al., 1998; Nagahara et al., 1999). These proteins have also been reported to include transcription factors, such as PDX-1 and Neurogenin (Kwon et al., 2005; Bendala et al., 2005).

Recently, one of the neurospecific transcription factors, NeuroD protein, has been demonstrated to be capable of transducing itself into pancreatic cells (Noguchi et al., 2005). NeuroD is also important for pancreatic islet morphogenesis and insulin gene transcription, and these authors attributed the transduction ability of NeuroD to the presence of arginine- and lysine-rich sequences that fulfilled the functions of PTD.

In this report, we show that NeuroD2 could be transduced into the N1E-115 mouse neuroblastoma cell line using this highly basic domain and that this protein was capable of inducing neural differentiation.

2. Materials and methods

2.1. Construction of expression vectors

Expression vectors for NeuroD2 and its variants were constructed as follows: the mouse NeuroD2 gene was obtained from the plasmid we described previously (Mie et al., 2003). The variants, $ND2_{\Delta 100–115}$ (lacking including nuclear localization signal (NLS) region), $ND2_{\Delta 123–134}$ (lacking the basic region of bHLH) and $ND2_{\Delta 100–134}$ (lacking NLS region and basic region) were amplified by the polymerase chain reaction (PCR). These genes were then inserted to the modified pET32c vector from which thioredoxine tag coding sequence had been deleted and the resulting plasmids were designated pET-NeuroD2, pET-$ND2_{\Delta 100–115}$, pET-$ND2_{\Delta 123–134}$ and pET-$ND2_{\Delta 100–134}$, respectively.

2.2. Expression and purification of proteins

The constructed plasmids were transfected into *E. coli* BL21 (DE3). To express the protein, transfected BL21 (DE3) cells were cultured for 3 h at 30 °C with 0.5 mM isopropyl-β-thiogalactopyranoside and dissolved in BugBuster agents (Novagen). After removing the supernatant, the insoluble fraction was dissolved in phosphate-buffered saline (PBS) with 4 M urea, and the protein component was purified using HIS-Select™ Nickel Affinity Gel (Sigma). After purification, all proteins were dialyzed against PBS to remove urea and imidazole agents. The protein concentration was adjusted to 0.2 μM after measurement using a BCA protein assay kit (PIERCE). Purified proteins were analyzed by 12% SDS-PAGE under reduced conditions and stained with CBB. These proteins were stored in PBS at 4 °C.

2.3. Protein modification with fluorescence and microscopy observation

Proteins were modified with Oregon Green 488 carboxylic acid and succinimidyl ester (Molecular Probes) as per manufacturer's instructions. The uptake of Oregon green-modified proteins into cells was analyzed using a confocal microscope (Olympus IX70 with FV300 system). Cells were grown on 35 mm glass-bottomed culture dishes to 20–30% confluence and observed 14 h after protein addition.