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Research Report

Antibody-mediated Hsp70 protein therapy

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ARTICLE INFO

Article history:
Accepted 13 March 2006
Available online 21 April 2006

Keywords:
Hsp70
Protein therapy
Protein transduction
Tat
Neuroprotection
Antibody

ABSTRACT

Intracellular Hsp70 provides cytoprotection against a variety of stressful stimuli, and an effective means of increasing intracellular Hsp70 levels could prove beneficial in the prevention and treatment of a variety of human diseases. A novel protein transduction domain consisting of the single chain Fv fragment of an anti-DNA antibody known to penetrate into living cells and tissues, mAb 3E10, has recently been used to deliver functional proteins to cells. The ability of the single chain Fv fragment to deliver Hsp70 into living cells was tested by generating an Fv-Hsp70 fusion protein. Fv-Hsp70 was produced as a secreted protein in both COS-7 cells and the methylotropic yeast strain *Pichia pastoris* and was shown capable of penetrating into COS-7 cells and primary rat cortical neurons. Pre-treatment with Fv-Hsp70 protected both COS-7 cells and primary rat cortical neurons against subsequent exposure to hydrogen peroxide. These results provide the first evidence that the Fv fragment of mAb 3E10 is capable of delivering proteins to neurons and indicate its potential in the development of Hsp70 protein therapy.

1. Introduction

Hsp70 is a member of the heat shock family of proteins that play key roles in cytoprotection by refolding damaged proteins and reducing protein aggregation during periods of cell stress. Augmentation of intracellular Hsp70 content exerts protective effects in cardiovascular disease, neurodegenerative disease, diabetes, and trauma (Soti et al., 2005). Investigators have used a variety of techniques in attempting to increase intracellular levels of Hsp70, including stress induction (Jiang et al., 2005), application of small molecule inducers of Hsp70 (Polakowski et al., 2002), and Hsp70 gene therapy (Yenari et al., 2005; Badin et al., 2006; Zheng and Yenari, 2006). Direct delivery of Hsp70 protein to cells is an emerging technique (Lai et al., 2005; Wheeler et al., 2003).

The success of intracellular protein therapy is critically dependent upon the ability of therapeutic proteins to cross the cell membrane and enter into cells. The amphipathic nature of the cell membrane presents a major barrier to proteins that has only recently been circumvented by protein transduction domains. The most frequently utilized protein transduction domains, termed cell-penetrating peptides (CPPs), include the HIV-1 Tat peptide, HSV VP-22 peptide, and simple poly-arginine peptides (Deshayes et al., 2005; Wadia and Dowdy, 2005; Phelan et al., 1998; Matsui et al., 2003). Tat-Hsp70 fusion proteins penetrate into living cells and protect neurons from

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doi:10.1016/j.brainres.2006.03.025

nitrosative and excitotoxic stress (Lai et al., 2005) and HSF null fibroblasts from hyperoxia and lethal heat shock (Wheeler et al., 2003). While effective in vitro, the highly basic CPPs may induce a potent toxic or inflammatory response that limits their viability as delivery vehicles for protein therapy in human disease (Lai et al., 2005; Toborek et al., 2003; Pu et al., 2003; Moulton et al., 2003).

The use of an autoantibody fragment as a delivery vehicle for protein therapy has recently been reported and reviewed (Hansen et al., 2005). Briefly, the Fv fragment of mAb 3E10, an anti-DNA autoantibody that penetrates into the nuclei of living cells, has been used to deliver functional proteins such as p53 (Weisbart et al., 2004a) and micro-dystrophin (Weisbart et al., 2005) into a variety of cells. Antibodies can induce self-tolerance and should not promote inflammation (el-Amine et al., 2002; Zambidis and Scott, 1996). The Fv fragment may therefore present a more acceptable protein transduction vehicle compared to the CPPs. The experiments detailed in this study were designed to test the ability of the Fv fragment to penetrate into neurons and to carry cytoprotective quantities of Hsp70 into living cells.

2. Results

2.1. *An Fv-Hsp70 fusion protein secreted from COS-7 cells and penetrated into COS-7 cell nuclei*

An Fv-Hsp70 fusion protein was designed with the Fv fragment located N-terminal to the Hsp70 protein. Myc and His_6 tags were placed between Fv and Hsp70 to aid in detection and purification of the fusion protein (Fig. 1A). The mammalian expression vector pSG5-Fv-*hsp70* or pSG5-Fv was transfected into COS-7 cells by electroporation. Seventy-two hours after transfection, COS-7 cells were examined by staining with the anti-myc antibody to determine the intracellular localization of the proteins. Cytoplasmic and nuclear staining was observed in COS-7 cells transfected with either pSG5-Fv or pSG5-Fv-h*sp70* (Fig. 1B). Cells exhibiting nuclear staining localized in a circumferential pattern around cells stained primarily in the cytoplasm (Fig. 1B). It has been reported that Ab fragments of mAb 3E10 produced in COS-7 cells must be secreted from cells before they penetrate into cell nuclei. Ab fragments produced as intracellular, non-secreted proteins in COS-7 cells accumulate in the cytoplasm and do not translocate into the nucleus (Zack et al., 1996). The Ab fragments may require post-translational modification in the Golgi complex to attain optimum transduction efficiency or may require specific interactions with the external surface of the cell membrane to penetrate into the nucleus. The precise mechanism of nuclear penetration by Fv has not been resolved, but recognition of the importance of secretion distinguishes COS-7 cells producing cytoplasmic Fv from COS-7 cells with transduced intranuclear Fv. The intranuclear localization of Fv-Hsp70 in COS-7 cells surrounding cells producing cytoplasmic Fv-Hsp70 therefore demonstrated COS-7 cell secretion of Fv-Hsp70 protein into the media and transduction of Fv-Hsp70 into the nuclei of surrounding COS-7 cells.

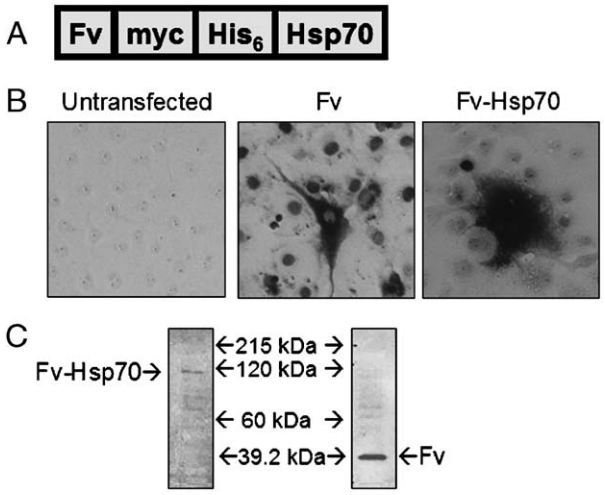

Fig. 1 – An Fv-Hsp70 fusion protein secreted from COS-7 cells and penetrated into COS-7 cell nuclei. (A) The Fv-*hsp70* constructs encode a protein comprised of the Fv fragment, myc and His_6 tags, and the Hsp70 protein. (B) COS-7 cells were transfected with either pSG5-Fv or pSG5-Fv-*hsp70* expression constructs. Seventy-two hours after transfection, COS-7 cells were fixed and stained with the anti-myc antibody. Sham transfected cells showed an absence of staining, while cells transfected with either pSG5-Fv or pSG5-Fv-*hsp70* exhibited cytoplasmic and nuclear staining. Cells producing Fv or Fv-Hsp70 stained in the cytoplasm and surrounding cells penetrated by Fv or Fv-Hsp70 stained in the nucleus. 200× magnification. (C) Lysates of COS-7 cells transfected with pSG5-Fv or pSG5-Fv-*hsp70* were subjected to Western analysis using the 9E10 anti-myc antibody and demonstrated production of full-length Fv and Fv-Hsp70 proteins.

To verify that COS-7 cells produced full-length Fv or Fv-Hsp70, cell lysates were prepared and subjected to SDS-PAGE and Western analysis using the anti-myc antibody. Western blotting demonstrated synthesis of full-length Fv with a molecular weight of approximately 30 kDa and full-length Fv-Hsp70 protein with a molecular weight of approximately 120 kDa (Fig. 1C). Significantly greater quantities of Fv than Fv-Hsp70 were produced by the COS-7 cells.

Having shown transduction of Fv-Hsp70 into COS-7 cell nuclei, efforts next focused on testing the biological activity of Fv-Hsp70. The amounts of Fv-Hsp70 produced by transient transfection of COS-7 cells were inadequate for pursuing further experiments. Instead, Fv-Hsp70 was produced in the methylotropic yeast strain *Pichia pastoris*.

2.2. *Fv and Fv-Hsp70 proteins secreted from P. pastoris*

The yeast expression vector pPICZαA-Fv or pPICZαA-Fv-*hsp70* was electroporated into the X-33 strain of *P. pastoris*, and the X-33 cells expressed and secreted significant quantities of Fv or Fv-Hsp70 protein. SDS-PAGE and Western analysis of purified Fv revealed full-length Fv at the expected molecular weight of 30 kDa. Analysis of purified Fv-Hsp70 revealed two primary protein bands of approximate molecular weights of 120 kDa