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Pigpen is a cellular binding protein of therapeutic oligonucleotides

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Background

Understanding the mechanism of oligonucleotide (ON) uptake and cellular distribution is important for rational design of ON-based therapeutic strategies. The aim of this study was to investigate the possible relationship between cellular distribution of ON and the protein pigpen.

Methods

In vitro *interaction of ON with the protein pigpen was detected using mass spectrometry. Cellular distribution of pigpen and co-localization of pigpen with ON was studied by fluorescence microscopy in endothelial YPEN and microglial N9 cells.*

Results

Pigpen had similar distribution patterns in endothelial YPEN and microglial N9 cells. Pigpen was localized to the cytoplasm of both cell types. In addition, pigpen distributed to nuclei, excluding the nucleoli, and concentrated along the nuclear membrane and plasma membrane. Intensely stained foci were only observed in the nucleus and cytoplasm of YPEN cells. Although co-localization of pigpen with phosphorothioate (PS) ON was not observed for first hour after ON uptake, co-localization was observed 8 h later.

Discussion

These data suggest that pigpen binds therapeutic ON and thus might contribute to ON cellular distribution.

Keywords

binding proteins, cellular localization, oligonucleotides, pigpen.

Introduction

Understanding the mechanisms of oligonucleotide (ON) uptake and cellular distribution is important for rational design of ON-based therapeutic strategies. Receptor-mediated endocytosis is considered to be the major mechanism for foreign ON uptake [1–3]. Inside cells, ON may localize to the nucleus or shuttle between cytoplasm and nuclei [4,5], which might be the result of their interaction with different cellular proteins. Recently, our group identified several proteins that bound to ON *in vitro* by mass spectrometry sequencing [6]. Accidentally, pigpen was identified binding ON in microglial N9 and glioblastoma C6 cells, which indicated pigpen might contribute to therapeutic ON uptake and/or cellular distribution.

Pigpen is a 67-kDa protein and a member of the EWS family. Sequence analysis has identified many important domains of pigpen, including a 'greek key' hairpin loop motif, two RNA-binding domains, seven copies of a degenerate glutamine-rich hexapeptide repeat, which is also found in the transactivation domains of several transcription factors, a consensus tyrosine phosphorylation site, which may be important for the redistribution of this protein during mitosis, and a carbohydrate-binding region [7]. Immunofluorescence staining has shown pigpen homogeneously distributed to the nucleoplasm and concentrated along the nuclear membrane and nuclear granules in proliferating endothelial cells [7,8]. The expression of pigpen in endothelial cells is higher than in other cell types, and its expression in proliferating cells is higher than in quiescent cells [9,10]. Pigpen has also been observed to be highly expressed in tumor microvessels of experimental rat brain glioblastoma but not in established peritumoral vessels [11]. Pigpen has high identity with the *FUS* gene

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product TLS (translocated in liposarcoma), a new member of the EWS family of proteins [9].

We have studied the cellular distribution of pigpen and the co-localization of pigpen with foreign phosphorothioate (PS) ON in microglial N9 cells. Microglial cells are the brain's effector cells of the innate immune system and play a key role in many central nervous system pathologies. Furthermore, pigpen has been identified as a binding protein for PS ON in microglial N9 cells. The interaction of pigpen with foreign ON was also studied in endothelial YPEN cells, because pigpen is highly expressed in endothelial cells. Most known data of pigpen are from endothelial cells, in addition to reported pigpen expression in YPEN [11].

Methods

Synthetic ON and Ab

All ON were from MWG-Biotech AG (Ebersberg, Germany). Biotin-labeled full PS ON were used for protein purification (CpG3, 5′-TCCATGACGTTCCTGATCGT-3′; CpG-inverse3, 5′-TCCATGAGCTTCCTGATGCT-3′). FITC-labeled PS CpG-inverse3 was used for analysis of intracellular distribution.

Pigpen Ab was a gift from Dr MC Alliegro (Department of Cell Biology and Anatomy, Louisiana State University Health Sciences Center, New Orleans, LA, USA). This Ab is directed against a region in the c-terminus, touching upon the RNP-CS region and greek key motifs. Secondary Ab, $F(ab')_2$ fragments labeled with FITC or rhodamine, was from CHEMICON International Ltd (Hofheim, Germany).

Cell cultures

Murine microglial N9 cells, murine endothelial YPEN cells and rat glioblastoma C6 cells were from ATCC (Wesel, Germany) and were cultured in RPMI-1640 with 10% heat-inactivated FCS, with penicillin and streptomycin at 100 U/mL (Gibco, Grand Island, NY, USA), at 37°C in 5% CO_2.

ON-mediated protein purification

N9 or C6 cells cultured in flasks were rinsed with ice-cold PBS twice and then collected with a scraper. Cells were sonicated and incubated in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma, Munich, Germany) for 2 h on ice. Lysates were cleared by centrifugation at 14 000 *g* for 5 min at 4°C. An aliquot of the supernatants was used for protein purification.

Magnetic streptavidin microbeads (Dynal Biotech ASA, Oslo, Norway) were coated with biotin-labeled ON by incubation at room temperature for 30 min. Subsequently, the lysates were incubated with ON-coated magnetic microbeads, in the presence of a 100-fold excess of tRNA as an unspecific competitor in selection buffer, for 30 min on ice. After washing five times, proteins were removed from ON-coated beads by heating in the loading buffer, analyzed by 10% PAGE and detected by staining with Coomassie blue. The bands were then analyzed by tryptic digestion and mass spectrometry [11].

Protein identification

In gel, tryptic digestion was performed as described elsewhere[12] and modified as outlined below. Briefly, the protein band was excised from the gel, fully destained, and digested for 3 h with porcine trypsinase (sequencing grade, modified; Promega, Mannheim, Germany) at a concentration of 67 ng/μL in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1–2 μL were left, and the peptides were purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA, USA). MALDI-TOF analysis from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target using the fast evaporation method [13] was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a N_2 337 nm laser, gridless pulsed ion extraction, and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionization mode at 23 kV.

Sequence verification of some fragments was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, UK) equipped with a nanoflow electrospray ionization source. Gold-coated glass capillary nanoflow needles were obtained from Protana (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science [14].