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MAPPING OF CALMODULIN-BINDING SITE ON THE C-TAIL OF TRPC6 CHANNEL

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Transient receptor potential channel TRPC6 is non-selective calcium permeable cation channel expressed in many cell types, including sensory receptor cells. TRPC6 consists of four subunits with six membrane-spanning domains and intracellular N- and C-terminal. Calmodulin (CaM) takes part in the calcium-dependent regulation of many proteins, including ion channels. There was identified one CaM-binding site on the C-tail of TRPC6. The aim of this study is to map in detail C-terminal region of mouse TRPC6 that is capable of interacting with CaM using *in-vitro* binding assays. The part of sequence of the C-tail (amino acids 801–878) was subcloned into pET15b or pET42b expression vectors and used as a template for site-directed mutagenesis. There were performed mutations of several amino acid residues that could potentionally distrupt CaM binding. These residues were chosen on the basis of three-dimensional computer model. All fusion proteins were expressed in *E. coli* BL21 and purified using nickel-chelating sepharose. The homogeneity of the purified recombinant proteins was confirmed by SDS-PAGE electrophoresis and mass spectrometry. The ability of binding of the protein (amino acids 801–878) and its mutants to CaM was tested by fluorescent anisotropy measurements using CaM Alexa Fluor 488 fluorescent probe. Our results show that amino acids R852, Y854, K856, M858, K859, R860, L861, K863, R864 and L867 participate in CaM binding on C-termini of TRPC6.

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SIRNA-MEDIATED KNOCKDOWN OF Bcl-2 IN HUMAN GLIOBLASTOMA CELL LINES INCREASED TAXOL-MEDIATED APOPTOSIS

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Use of small interfering RNA (siRNA) is an incredibly powerful tool to knockdown the mRNA level and thus protein level of a target gene. Induction of apoptosis with a chemotherapeutic agent offers a potentially useful strategy for treatment of various cancers including glioblastoma. Taxol (also known as paclitaxel) is a potent anti-neoplastic drug that strongly binds to the β-subunit of tubulin to prevent tumor cell division and promote cell death. Anti-apoptotic molecule Bcl-2 is up-regulated in glioblastomas to protect them from apoptosis. The aim of our present investigation was to down-regulate Bcl-2 level for increasing apoptosis in glioblastoma cells with a low dose of taxol. We used Bcl-2 siRNA to knockdown the cognate mRNA and subsequently protein levels in two highly invasive glioblastoma U138MG and U251MG cell lines. The cells were treated with either Bcl-2 siRNA (100 nM) or taxol (100 nM) or both and for 72 h. TUNEL assay and FACS analysis were used for determination of amounts of apoptosis. Fluorometric assays showed that increase in calpain and caspase-3 activities enhanced apoptosis. *In situ* immunofluorescent labelings indicated co-localization of active calpain and caspase-3 in apoptosis. Western blotting demonstrated dramatic increases in levels of TRADD, FADD, Bad, Bax, active caspases, PARP cleavage, and DFF40 during apoptosis. The events related to apoptosis were more prominent in combination therapy than either treatment alone. Thus, we demonstrated that treatment with combination of Bcl-2 siRNA and taxol was highly effective for increasing apoptosis in U138MG and U251MG cells due to induction of calpain and caspase mediated signaling pathways.

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COORDINATED GANGLIOSIDE BIOSYNTHESIS IN PRIMARY HIPPOCAMPAL NEURONS: ANALYSIS BY RNAI GENE SILENCING

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Gene knockdown with siRNA was used to investigate the possibility that glycosyl transferases act in a coordinated manner in the synthesis of complex gangliosides. We have developed a method for culturing primary neurons from neonatal rat hippocampus, in which genes for glycosyltransferases are suppressed by RNAi transfection. These cells differentiate and synthesize a normal complex ganglioside pattern, but siRNA against sialyltransferase II (ST2) reduces mRNA levels for this enzyme by 68%, as confirmed by quantitative PCR (qPCR). However, qPCR also confirmed suppression of expression of the gene encoding *N*-acetyl galactosaminyltransferase (GNT) by 67% in the same experiment. TLC analysis of ganglioside patterns 48 h after knockdown shows total blockage of complex ganglioside synthesis, including those that require GNT. These results are consistent with similar observations reported by Bieberich *et al. Biochem.* **41** 11479, (2002) for immortalized F-11A neuroblastoma cells. Our results are consistent with the possibility that ST2 and GNT act in a coordinated fashion as part of an enzymatic complex. Another possibility is that all complex gangliosides originate from a GD3 precursor, with mono-sialogangliosides deriving secondarily from sialidase action against multi-sialoganglioside precursors.

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