Association of NGF receptors with membrane rafts in PC12 cells

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ABSTRACT

Nerve Growth Factor (NGF) signal transduction is involved in the survival, differentiation and maintenance of neurons through the receptors TrkA and $p75^{NTR}$. These receptors activate downstream protein kinase cascades that regulate cell survival. NGF binding to TrkA promotes cell survival, however NGF binding to the low-affinity receptor, $p75^{NTR}$ can lead to cell death in the absence of TrkA. Therefore the interaction of these two receptors and their downstream pathways are very important for determining cell survival. Recent studies have shown that many receptors and their associated downstream proteins have been found in membrane rafts, areas of the plasma membrane enriched in sphingolipids and cholesterol. To investigate the presence of the NGF receptors and downstream signalling proteins in these rafts, we have devised a method of cellular fractionation and detergent extraction quite different from those used previously. Mechanical permeabilisation separated the cytosolic components of PC12 cells. Non-ionic detergent extraction was used to solubilise the majority of the plasma membranes, leaving the detergent-insoluble membranes and cytoskeleton. Equilibrium flotation gradients were used to separate the membrane rafts from other detergent-insoluble material such as the cytoskeleton. Using these methods, we found that not only are TrkA and $p75^{NTR}$ present in rafts, but also the downstream signalling protein ERK1 and the cytoskeletal protein, tubulin. In addition to plasma membrane rafts, we have isolated detergent-insoluble intracellular membranes from the endoplasmic reticulum and Golgi. NGF binding, *in vitro* reactions with an ATP regenerating system and the addition of ganglioside GM1 to the cells, have been found to have a large effect on the raft association of both TrkA and $p75^{NTR}$. These results indicate an important role for membrane rafts in NGF signalling through its receptors TrkA and $p75^{NTR}$, and suggest a model in which signalling centres form around rafts and microtubules.

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ABBREVIATIONS

Akt/PKB	Protein kinase B (serine/threonine kinase)
ATP	Adenosine triphosphate
BB	Bud buffer
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CNS	Central nervous system
CPM	Counts per minute
Da	Dalton
DAG	Diacylglycerol
ddH_20	Double distilled water
DIGs	Detergent-insoluble glycolipid-rich domains
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRM	Detergent-resistant membranes, TX100-insoluble pellet (10,000xg)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase (ERK1 = p44 MAPK, ERK2 = p42 MAPK)
Flot	Flotillin
FRET	Fluorescence resonance energy transfer
GD1	Disialoganglioside
GDP	Guanosine diphosphate
GM1	Monosialoganglioside
GM2	Monosialoganglioside
GM3	Monosialoganglioside
Gmix	Ganglioside standard mixture
GPI	Glycosylphosphatidylinositol
Grb-2	SHC binding protein

GSK-3	Glycogen synthase kinase-3
GSLs	Glycosphingolipids
GTP	Guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IP_3	Inositol 1,4,5 trisphosphate
JNK	c-Jun amino-terminal kinase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MβCD	Methyl-β-cyclodextrin
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NT	Neurotrophin
NT-3	Neurotrophin-3
P1	1,000xg pellet - cell membranes, cytoskeleton, large organelles
P1M	10,000xg supernatant - solubilised membranes
p70S6K	p70 ribosomal protein S6 kinase
$p75^{NTR}$	75 kDa neurotrophin receptor
PBS	Phosphate-buffered saline
PC12	Rat adrenal pheochromocytoma cell line
PEE	PBS with EDTA and EGTA
pERK	hyperphosphorylated ERK1 (~46 kDa)
PGB	PBS with glucose and BSA
PI 3,4-P_2	Phosphatidylinositol 3,4-bisphosphate
PI 4,5-P_2	Phosphatidylinositol 4,5-bisphosphate
PI3-kinase	Phosphatidylinositol 3'-kinase
PKBKs	Protein kinase B kinases
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
PM	Plasma membrane
PMSF	Phenylmethylsulfonyl fluoride
Raf	MAPK/ERK kinase kinase

Ras	GTP binding protein
RTA	anti-rat TrkA antibody
S1	1,000xg supernatant - small organelles, cytosol
SAPK	Stress-activated protein kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHC	TrkA binding protein, src homology containing protein
SOS	Guanine nucleotide exchange factor
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TrkA	140 kDa tyrosine receptor kinase A
Tween-20	Polyoxyethylenesorbitan monolaurate
TX100	Triton X-100
v/v	volume/volume
w/v	weight/volume

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