Apparent presence of Ser133-phosphorylated cyclic AMP response element binding protein (pCREB) in brain mitochondria is due to cross-reactivity of pCREB antibodies with pyruvate dehydrogenase

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Introduction

The cyclic AMP response element binding protein (CREB) is an ubiquitously and constitutively expressed transcription factor of the basic/leucine zipper family that recognizes a DNA consensus sequence called CRE (cyclic AMP/Ca2+ response element). Its ability to activate transcription of the regulated gene critically depends on phosphorylation of a serine residue number 133 in its transactivation domain, which is done by variety of protein kinases in response to elevation of intracellular calcium, cAMP or neurotrophin signaling.

With over 5000 reports related to CREB in Medline to date this protein has also become one of the most studied transcription factors. In neuroscience the CREB has been widely implicated in the synaptic plasticity underlying long-term memory consolidation, and also in the neuronal pro-survival signaling pathways.

The prevailing view is that CREB is permanently and exclusively present in the cell nucleus. However, there have been several mutually controversial reports describing an extranuclear localization of CREB. Specifically, synaptic, cytosolic, and in particular mitochondrial localization was reported.

Here we present results of our own study on subcellular localization of CREB.

Methods

The subcellular localization of CREB was examined by means of:

- Subcellular fractionation of adult rat brain cortex followed by Western imunoblotting with CREB/pCREB antibodies and gel shift with CRE probe. Suitable nuclear, mitochondrial and synaptic markers also employed.

- Immunofluorescence staining and confocal microscopy of cultured rat brain neuronal and astroglial cells

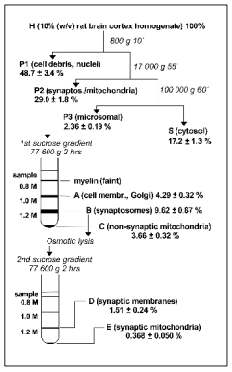
- Immunoprecipitation of CREB or pCREB IR from nuclear or mitochondrial fractions, followed by Western blotting analysis and identification of precipitated proteins by MALDI-TOF mass spectrometry

The following commercial CREB/pCREB antibodies (Abs) were used:

Phospho-CREB (Ser133) Ab, Cell Signalling Technology CST #9191)
Phospho-CREB Ab from Upstate Biotechnology (#06-519)
CREB antibody (against an epitope at the CREB C-terminus), CST (#9192)
C-21 CREB Ab (against CREB DNA-binding domain), Santa Cruz Biotechnology (sc-186) .
HmCREB antibody (from "home-made") was raised in Y. Yoneda's laboratory against peptide KRREILSRRPSYRKC (residues 123-136 of mouse or rat CREB, encompassing unphosphorylated Ser133, and affinity-purified using the same peptide.

(pCREB... CREB phosphorylated at Ser133, IR... immunoreactivity, MALDI-TOF... matrix-assisted laser desorption ionisation time-of-flight)

1) Subcellular fractionation procedure



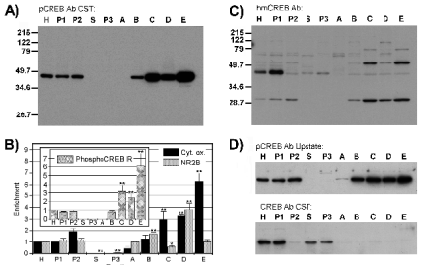
Fractions obtained:

H (original homogenate)
P1 (nuclear fraction, but also containing a lot of mitochondria from cell debris)
P2 (crude mitochondria/synaptosomes)
S (cytosol)
P3 (microsomal)
A (probably cell membranes, Golgi)
B (synaptosomes)
C (non-synaptic mitochondria)
D (synaptic membranes, but also containing a lot of synaptic mitochondria)
E (synaptic mitochondria)

In each fraction measured:

Protein concentration and yield
Western blotting for
CREB,
pCREB,
NMDA receptor 2B subunit
Cytochrome oxidase activity
Gel shift for CRE DNA binding activity

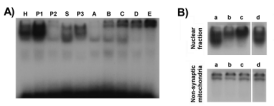
2) pCREB IR is prominent in mitochondrial fractions, but CREB IR is not



A), C), D): representative Western blots of the subcellular fractions probed with various pCREB and CREB antibodies as indicated. B): Densitometric evaluation of the pCREB Ab CST IR (inset) in comparison to distribution of NMDA receptor 2B subunit IR (synaptic marker) and azide-inhibitable cytochrome oxidase enzyme activity (mitochondrial marker). Mean ±SD, N=4 independent experiments, only for cytochrome oxidase N=2 independent experiments. *... p<0.05, **... p<0.01 vs. original homogenate), one-way ANOVA with Dunnett's post-test.

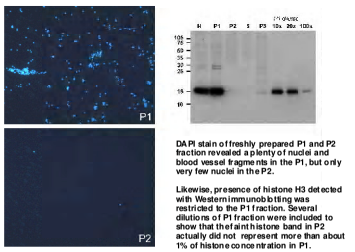
The signal with two different pCREB Ab's was strong in mitochondria-enriched fractions (C, D, E in particular), and quantitative distribution of pCREB IR correlated with the mitochondrial marker rather than synaptic one. In contrast, the band detected with two CREB antibodies against different epitopes was seen mostly in nuclear fraction, some in S and P3, but almost absent from mitochondrial fractions.

3) Gel shift: mitochondrial/synaptic CRE DNA binding activity different from nuclear CREB



Gel shift with 32P-labelled CRE probe performed with all the subcellular fractions, for nuclear and mitochondrial fractions also including supershift with various CREB/pCREB Ab's as indicated).
The dominant lower band appearing in the nuclear fraction was supershifted by two CREB Abs which qualifies this band as authentic CREB. In contrast, the CRE- binding activity in mitochondrial/ synaptic fractions differed in electrophoretic mobility and was unaffected by CREB Ab's.

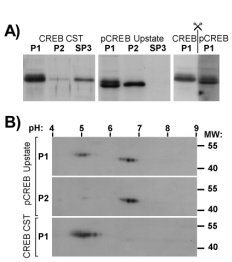
4) Nuclear contamination of P2 fraction was low but detectable



DAPI stain of freshly prepared P1 and P2 fraction revealed a plenty of nuclei and blood vessel fragments in the P1, but only very few nuclei in the P2.

Likewise, presence of histone H3 detected with Western immunoblotting was restricted to the P1 fraction. Several dilutions of P1 fraction were included to show that the faint histone band in P2 actually did not represent more than about 1% of histone concentration in P1.

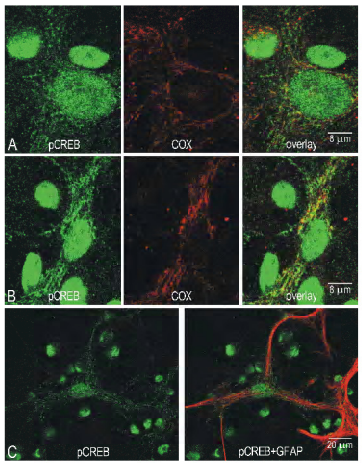
5) Electrophoretic mobility of mitochondrial pCREB IR markedly differed from nuclear pCREB/CREB IR



A): The CREB IR on Western blots detected with the CREB Ab CST in the nuclear fraction consistently appeared as a double band, likely representing two CREB splicing variants, alpha (the upper band) and delta (the lower band). All other fractions apparently contained only delta CREB. The blots detected with pCREB Ab Upstate also displayed a non-constant double band in the nuclear P1 fraction, while in the mitochondrial P2 fraction there was always a single band with position corresponding to the P1 lower band.
Most importantly, the positions of nuclear double bands detected with CREB Ab CST and pCREB Ab Upstate did not correspond to each other. Rather, the lower band detected with CREB Ab CST corresponded to the upper band detected with pCREB Ab Upstate.

B) 2-D electrophoresis of P1 (upper panel) and P2 (middle panel) fractions followed by Western immunoblotting with pCREB Ab Upstate. The P1 blot then subjected to stripping procedure, developed with secondary antibody to verify complete removal of the pCREB Ab, and reprobed with the CREB Ab CST (bottom panel).
2-D electrophoresis resolved the nuclear and mitochondrial pCREB IR's into two spots with such a big difference in apparent isoelectric points, that they must be completely different proteins. The mitochondrial pCREB IR in fact represents another mitochondrial protein, which cross-reacts with the pCREB Ab's.

6) pCREB Ab's stained mitochondria, in addition to nuclei, of glial cells in primary cortical cultures

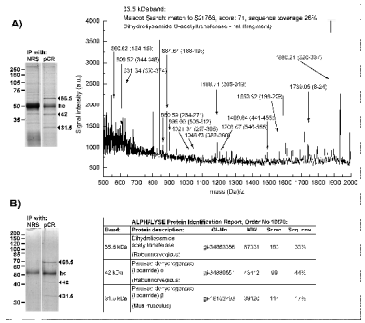


The primary neuronal cultures were made of new-born rat cortices, cytosine arabinoside added at day 2, and cultures used for immunofluorescence staining at day 3-6. Most of the cells were neurones, while glial elements accounted for less than 5 %. Antibodies against pCREB/ CREB, cytochrome oxidase (COX) or astrocytic marker GFAP were used for staining, and the preparations were examined by confocal microscopy. pCREB antibody Upstate stained nuclei of all cells, but also mitochondria of glial elements, where it co-localized with COX. The mitochondrial signal was evident even in unstimulated cultures (A), but was stronger when the cultures were challenged with forskolin (activator of protein kinase A) prior to fixation (B, C). If CREB Ab was used for staining, only faint nuclear signal could be obtained in this experimental system.

The pyruvate dehydrogenase (PDH) consists of three major components: pyruvate dehydrogenase (decarboxylase (E1) (EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2) (EC 2.3.1.12) lipoamide dehydrogenase (E3) (EC 1.6.4.3.), with stoichiometry 2:030 alpha2beta2 tetramers + 6 homodimers of E3 + 60 copies of E2 (Swissprot entry P09061)

(Figure from Lodish et al. Molecular Cell Biology, 5th ed., W.H.Freeman Co., N.Y. 2004)

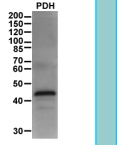
7) Three proteins precipitated by pCREB Ab from P2 fraction were identified as PDH



Successful immunoprecipitation of the 'false' pCREB IR from P2 was consistently associated with 3 Coomassie-detectable protein bands of apparent MW 65.5, 42 and 31.5 kDa. These 3 bands were conclusively identified by MALDI-TOF MS as the E2, E1 alpha subunit, and E1 beta subunit components, respectively, of the well-known mitochondrial enzyme complex pyruvate dehydrogenase (PDH)

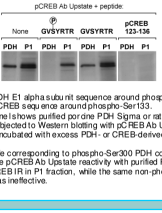
In experiment A) we achieved identification of the 65.5 kDa band (MALDI-TOF MS spectrum shown). B) is another experiment where immunoprecipitation procedure was improved and the three bands were sent to Alphalyse AS, (Odense, Denmark, http://www.pick-n-post.com), which offers the MALDI-TOF MS protein identification as a commercial service; on the right is summary of results provided by the Alphalyse.

8) pCREB Ab reacts with purified pyruvate dehydrogenase



Pyruvate dehydrogenase (PDH) purified from porcine heart (Sigma P-7032), subjected to Western immunoblotting with pCREB Ab Upstate: a single band of MW about 42 kDa (corresponds to the PDH E1 alpha subunit) is seen.

9) The cross-reacting epitope is phospho-Ser300 on PDH E1 alpha



The rat PDH E1 alpha subunit sequence around phospho-Ser300 is similar to CREB sequences around phospho-Ser133.
Bottom panel shows purified porcine PDH Sigma or rat brain P1 fraction subjected to Western blotting with pCREB Ab Upstate that had been pre-incubated with excess PDH- or CREB-derived peptides as indicated.
The peptide corresponding to phospho-Ser300 PDH completely blocked the pCREB Ab Upstate reactivity with purified PDH as well as 'false' pCREB IR in P1 fraction, while the same non-phosphorylated peptide was ineffective.

Conclusions

We have found a pCREB-like immunoreactivity in brain mitochondria, which, however, after careful scrutiny turned out to be pyruvate dehydrogenase, rather than authentic CREB.

This finding can explain most of the experimental evidence for mitochondrial localization of CREB, published previously; taken this into account there does not seem to be enough evidence for mitochondrial CREB localization.

The commercial antibodies directed against CREB phospho-Ser133 cross-react with phospho-Ser300 on E1 alpha subunit of pyruvate dehydrogenase. The antibodies should be used with caution and some of the previous data based on their usage may need to be revisited.

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