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CREB in Long-Term Potentiation in Hippocampus: Role of Post-Translational Modifications–Studies *In Silico*

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ABSTRACT

The multifunctionality of proteins is dictated by post-translational modifications (PTMs) which involve the attachment of small functional groups such as phosphate and acetate, as well as carbohydrate moieties. These functional groups make the protein perform various functions in different environments. PTMs play a crucial role in memory and learning. Phosphorylation of synaptic proteins and transcription factors regulate the generation and storage of memory. Among these is the cAMP-regulated element binding protein CREB that regulates CRE containing genes like *c-fos*. Both phosphorylation and acetylation control the function of CREB as a transcription factor. CREB is also susceptible to *O*-GlcNAc modification, which inhibits its activity. *O*-GlcNAc modification occurs on the same or neighboring Ser/Thr residues akin to phosphorylation. An interplay between these modifications was shown to operate in nuclear and cytoplasmic proteins. In this study computational methods were utilized to predict different modification sites in CREB. These in silico results suggest that phosphorylation, *O*-GlcNAc modification and acetylation modulate the transcriptional activity of CREB and thus dictate its contribution to synaptic plasticity. J. Cell. Biochem. 112: 138–146, 2011.

KEY WORDS: CREB-TRANSCRIPTION FACTOR; POST-TRANSLATIONAL MODIFICATIONS; HIPPOCAMPUS; TRANSCRIPTION; SYNAPTIC PLASTICITY

The contributions of post-translational modifications (PTMs) in memory and learning are well-recognized molecular events [Routtenberg and Rekart, 2005; Sunyer et al., 2008]. Modifications such as phosphorylation, palmitoylation, acetylation, and glycosylation of neuronal receptors, neurotransmitters, transcription factors, and kinases play very important roles in synaptic plasticity [Sunyer et al., 2008]. These different PTMs of proteins regulate the short-term and the long-term neuronal plasticity, the latter also requiring de novo protein synthesis.

Learning and memory differ in that learning is acquired information and memory is maintained information. The hippocampus is important for studying different types of memory such as spatial and recognition memory [Broadbent et al., 2004]. It is located in the medial temporal lobe and is divided into different areas, which processes the incoming signal. The information enters the hippocampus via the perforant path. The axons then synapse on cells in dentate gyrus, from where signaling is forwarded via axons (also called Mossy fibers) to the cornus ammonis 3 (CA3). CA3 sends axons called Schaeffer collaterals to cornus ammonis 1 (CA1). The CA1 forwards these signals through another set of fibers to the subiculum, which sends axons out of the hippocampus to different parts of brain [Sekino et al., 1997]. This process of memory acquisition (or storage) occurs via the formation of synapses. One of the main processes in memory acquisition involves long-term potentiation (LTP), a long-lasting connection between two neurons. In the hippocampus, as compared to the rest of the brain, new neurons are produced throughout life, and these neurons are liable to undergo LTP.

During LTP, an increased influx of calcium into the cell depolarizes the post-synaptic membrane, which activates different

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kinases such Ca^{2+}/calmodulin dependent kinase (CAMKII) and protein kinase A (PKA) [Boehm and Malinow, 2005]. These kinases phosphorylate different post-synaptic receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate-glutamate receptors (NMDARs) [Boehm and Malinow, 2005]. LTP can be divided into an early short-term LTP (E-LTP), and a late long-term LTP (L-LTP), where the L-LTP requires protein synthesis in addition to PTMs of pre-existing synaptic proteins to establish a long-term memory. Furthermore some of the newly synthesized proteins or transcription factors are responsible for the synaptic efficacy, and also contributes to long-term memory. The conversion of short-term memory into long-term memory depends on the activation of the transcription factor cAMP response element binding protein CREB [Korzus, 2003].

The CREB transcription factor controls the transcription of genes containing cAMP response element (CRE-elements) in promoters of inducible genes such as somatostatin [Montminy et al., 1986] and *c-fos* [Lee et al., 1998]. When CREB binds to the CRE element, it consequently becomes phosphorylated on Ser133 in its kinase-inducible domain (KID) [Lonze and Ginty, 2002]. Phosphorylated CREB allows recruitment of the acetyltransferase CREB-binding protein (CBP) or its highly related co-activator p300 [Vo and Goodman, 2001] that binds to phosphorylated CREB on Ser133 and participate in the process of transcription [Cardinaux et al., 2000]. Several kinases have been shown to phosphorylate CREB depending on the stimulus. Among these are protein kinases A and C (PKA and PKC), mitogen activated protein kinase (MAPK), Ca^{2+}/calmodulin-dependent kinase (CAMK) [reviewed in Johannessen et al., 2004].

The CREB transcription factor regulates a wide range of biological functions, such as spermatogenesis [Walker et al., 1994], circadian rhythms [Ginty et al., 1993] and memory [Mizuno et al., 2002]. It is well documented that CREB plays an essential role in late L-LTP, rather than in E-LTP, during memory acquisition in the hippocampus of vertebrates and invertebrates [Impey et al., 1996; Balschun et al., 2003; Sunyer et al., 2008]. Furthermore it has been suggested that phosphorylation of CREB may serve as a molecular marker for spatial memory in the hippocampus [Mizuno et al., 2002]. Phosphorylation of CREB occurs in CA1 and dentate gyrus neurons [Bernabeu et al., 1997; O'Connell et al., 2000; Mizuno et al., 2002]. Damage in the hippocampus may lead to different pathologies such as ischemia [Lipton, 1999; Doyle et al., 2008], Alzheimer's disease [Jin et al., 2004], and epilepsy [Schaub et al., 2007]. These various diseases have shown to affect the different areas of the hippocampus, but the CA1 region has been shown to be the most sensitive region [Lipton, 1999; Jin et al., 2004; Schaub et al., 2007; Doyle et al., 2008].

Different PTMs regulate the functions of CREB. Acetylation in the activation domain of CREB on Lys 91, 94, and 136 by CBP/p300 augments its transactivation potential [Lu et al., 2003]. Furthermore, the CREB transcription factor becomes phosphorylated first, followed by attachment of CBP/p300 [Parker et al., 1996] and finally acetylated [Lu et al., 2003]. This increases the activity of CREB and consequently transcription. An equally important and dynamic PTM, the *O*-GlcNAc modification, detectable in nearly all higher eukaryotic organisms [Comer and Hart, 2000], is also known to occur in CREB [Lamarre-Vincent and Hsieh-Wilson, 2003]. *O*-GlcNAc modification of CREB on putative sites residing in the (256–261) sequence environment may affect memory storage by influencing its transcriptional activity [Lamarre-Vincent and Hsieh-Wilson, 2003]. *O*-GlcNAc modification is a ubiquitous modification that is regulated by *O*-GlcNAc transferase (OGT: adds *O*-GlcNAc to protein backbone) and *O*-GlcNAcase (OGN: removes *O*-GlcNAc from protein backbone). OGT shows high flexibility in recognition of its substrates, and to date no consensus sequence has been found. *O*-GlcNAc modification compared to other *O*-glycosylation such as *O*-GalNAc modification is a single sugar residue modification, which occurs on the hydroxyl function of Ser/Thr. It regulates protein folding, localization and trafficking, solubility, antigenicity, biological activity, and half-life, as well as cell–cell interactions [Love and Hanover, 2005]. A complex relation between phosphorylation and *O*-GlcNAc modification on the same or neighboring residues, the Yin Yang sites, also occurs in several nuclear and cytoplasmic proteins [Wells et al., 2003]. Furthermore *O*-GlcNAc modification is abundant in synaptosomes, and is known to regulate synaptic proteins [Cole and Hart, 2001]. These alternative modifications of Ser/Thr residues very often result in changing the function of a protein.

It appears that different PTMs and their combinatorial regulation of the transcriptional activity of CREB play a crucial role in LTP in the hippocampus. We have identified in silico phosphorylation, *O*-GlcNAc modification, and Yin Yang sites on Ser/Thr residues in human CREB, and propose a novel mechanism in LTP in the hippocampus controlled by PTMs.

This study was performed to delineate the role of PTMs in the multifunctionality of proteins, particularly in vivo where large numbers of other proteins are present. Defining PTMs of proteins in vivo is difficult and also of uncertain functional relevance. The catalytic and associative functions of modified proteins are often influenced by a great number of proteins and other molecules; for example, blood alone contains more than 1,500 proteins [Sennels et al., 2007]. Consequently, the in vitro study of the structure–function relationship of a blood protein would hardly justify its functional relevance in vivo in the presence of numerous other proteins. The regulatory function of proteins depends on extremely few amino acids located in their conserved regions that cannot be detected by those state of the art experimental work. Therefore, in silico studies are essential to investigate the role of PTMs. Furthermore the functional switch of proteins is induced by numerous PTMs for a very short period of time, which is extremely difficult to determine experimentally. Computational methods are very useful to accurately predict transitory PTMs of proteins. These in silico methods provide a guideline with directed data that is useful for experimental studies to further assess the multifunctional behavior of proteins, and their role in learning and memory.

MATERIALS AND METHODS

SEQUENCE SELECTION

The sequence of CREB selected for the determination of potential phosphorylation and *O*-GlcNAc modification sites in *Homo sapiens*.

TABLE I. Different Species Selected for Multiple Alignment

Mammals	*Mus musculus*	AAB64015.1
	Bos taurus	P27925.2
Amphibia	*Xenopus tropicalis*	NP_998851.1
Aves	*Gallus gallus*	NP_989781.1
	Taeniopygia guttata	NP_001041721.1
Actinopterygii	*Ctenopharyngodon idella*	ABK55614.1
	Danio rerio	NP_957203.1

The sequence data were retrieved from the Swiss-Prot sequence database with accession no. P16220 [Boeckmann et al., 2003].

BLAST SEARCHING AND SEQUENCE ALIGNMENT

BLAST searching was carried out with the NCBI database (http://www.ebi.ac.uk/blastall/) [Altschul et al., 1997] of non-redundant sequences. The sequences were aligned with the ClustalW2 database [Thompson et al., 1994] (http://www.ebi.ac.uk/clustalw/). Four different classes of vertebrates were selected to determine the evolutionary conservation of the in silico predicted phosphorylation, *O*-glycosylation, Yin Yang and acetylation sites in human CREB (Table I).

PHORYLATION/*O*-GlcNAc MODIFICATION PREDICTION SITES

For prediction of potential phosphorylation and *O*-glycosylation sites in human CREB, NetPhos 2.0 (http://www.cbs.dtu.dk/services/Netphos/) [Blom et al., 1999] and YinOyang [Gupta and Brunak, 2002] were used, respectively. These two methods are neural network-based prediction methods. Neural networks are composed of a large number of highly interconnected processing elements (simulated neurons) working in parallel to solve a complex problem. In a neural network-based prediction method, networks are trained by sequence patterns of modified and non-modified proteins, so that they become able to recognize and predict a pattern with potential for modification in a new protein. Artificial neural networks receive many inputs and give one output as a result. NetPhos 2.0 was developed by training the neural networks with phosphorylation data from Phosphobase 2.0. The YinOYang 1.2 server [Gupta and Brunak, 2002] produces neural network predictions for *O*-GlcNAc attachment sites in eukaryotic protein sequences. This method is also able to predict potential phosphorylation sites and thus possible "Yin Yang" sites. A threshold value of 0.5 is used by Netphos 2.0 to determine potential phosphorylation sites, while the threshold value used by YinOYang 1.2 is variable, depending upon surface accessibility of the different amino acid residues. False negative sites were also identified, by coupling conservation status and modification potential of the two methods.

ACETYLATION PREDICTION SITES

The prediction of acetylation sites in human CREB, PAIL (Prediction of Acetylation on Internal Lysines) was utilized (unpublished).

RESULTS

PHOSPHORYLATION AND *O*-GlcNAc MODIFICATION IN HUMAN CREB

Prediction of phosphorylation in human CREB by Netphos 2.0 of Ser, Thr, and Tyr is presented in Figure 1A and in Table II. These results show a high phosphorylation potential in human CREB. As can be seen in Table II and Figure 2, nearly all the in silico phosphorylation sites are conserved in mammals, amphibians, birds, and Actinopterygii, and 10 phosphorylation sites have been experimentally verified by different groups [reviewed in Johannessen et al., 2004]. Prediction of potential *O*-GlcNAc modifications in human CREB by YinOYang 1.2 is given in Table II and Figure 1B. As can be seen the CREB protein shows a high potential for *O*-GlcNAc modification and most of the sites are conserved in mammals, amphibians, birds and, Actinopterygii.

YIN YANG SITES IN CREB

The prediction results of potential Yin Yang sites in CREB by YinOYang 1.2 is illustrated in Figure 1C and given in Table II. In some cases Ser/Thr residues show a very high potential for either *O*-GlcNAc modification or phosphorylation or show a potential very close to the specific threshold value predicted by the existing methods. When a residue shows a very high potential for phosphorylation and a potential for *O*-GlcNAc modification very close to the threshold value, it is considered a false negative Yin Yang site, as OGT and kinases may have an equal accessibility to modify such a residue. The in silico predicted Yin Yang site, Ser133, is a false negative Yin Yang site which is conserved in mammals, amphibians, birds, and Actinopterygii (Fig. 2, Table II). The other predicted Yin Yang sites, Ser117, 129, 260 and Thr18, 160, 167, 227 are conserved in mammals, amphibians, birds, and Actinopterygii (Fig. 2), except for Thr18 and 160 which are conservatively substituted in Actinopterygii and in *Danio rerio*, respectively.

ACETYLATION OF HUMAN CREB

All potential predicted Lys acetylation sites in human CREB (Lys91, 94, 123, 136, 155, 285, 292, 303, 304, 305, 309, 333, and 339) were found to be conserved in mammals, amphibians, birds, and Actinopterygii, except for Lys91 and 94 which are conserved in *H. sapiens* and *Mus musculus* with a deletion gap (DG) in the remaining sequence (see Fig. 2, Table III).

DISCUSSION

In the process of memory and learning, extracellular stimuli cause intracellular signaling and nuclear gene expression. Sustaining stable synapses during short-term memory requires PTMs, whereas additional gene transcription and protein synthesis are required for long-term memory [Kandel, 2001; Routtenberg and Rekart, 2005; Sunyer et al., 2008]. Ionotrophic glutamate receptors like NMDAR and AMPAR become activated upon binding to glutamate. NMDAR mediates influx of Ca^{2+} into the dendrite, leading to CAMKII and PKC activation [Zhu, 2003]. CAMKII promotes insertion of newly synthesized AMPAR into the synapse and it also phosphorylates CREB [Sunyer et al., 2008]. Furthermore, an increase in Ca^{2+} recruits adenylyl cyclase, thereby activating the cAMP-dependent kinase PKA. PKA migrates into the nucleus, where it phosphorylates CREB.

CREB controls about 4,000 target genes in the human genome by binding to CRE elements [Zhang et al., 2005], emphasizing its crucial role in transcriptional regulation. The CREB protein contains two

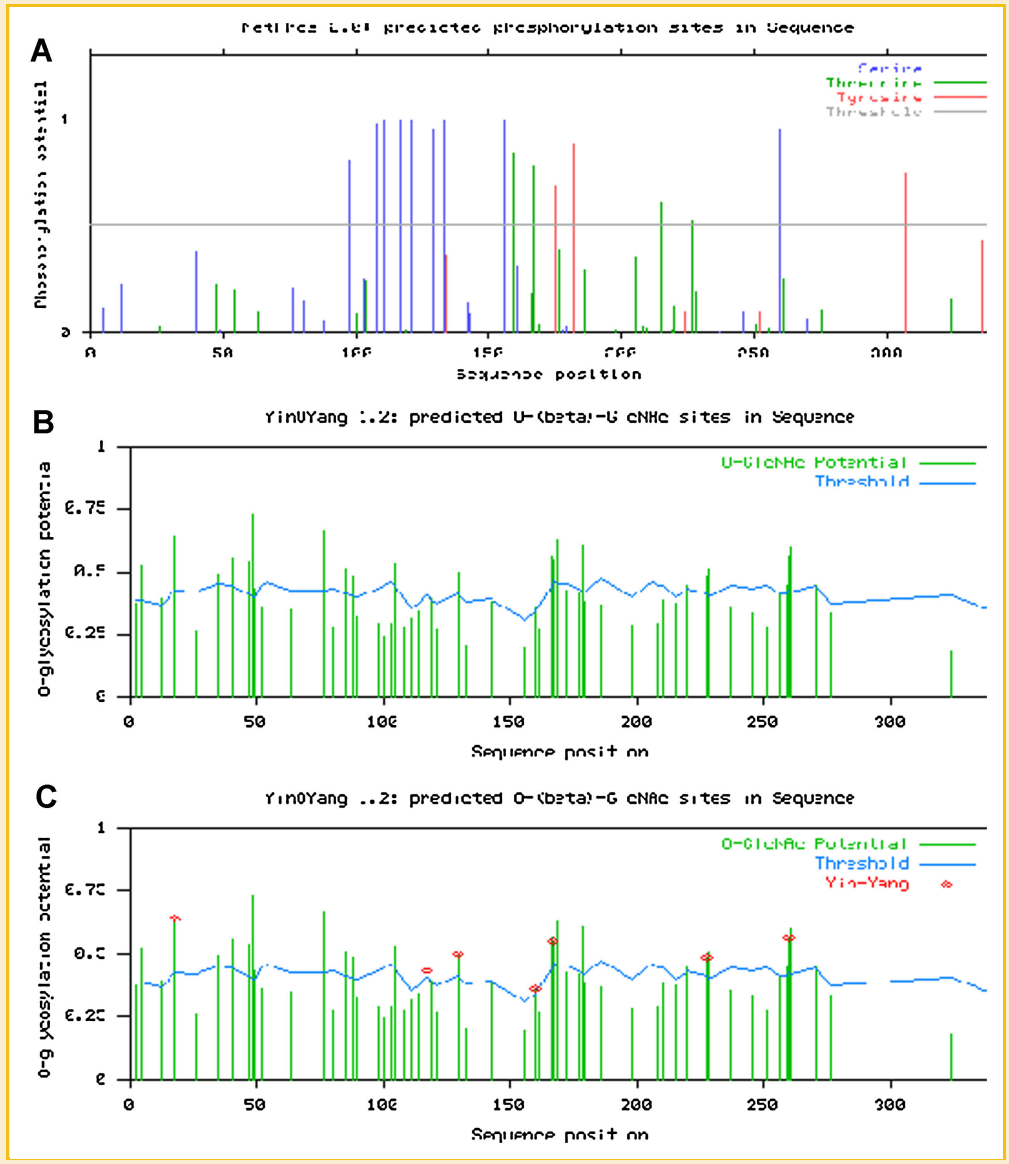

Fig. 1. A: Predicted potential sites for phosphate modification on Ser, Thr, and Tyr residues in human CREB transcription factor. The blue vertical lines show the potential phosphorylated Ser residues; the green lines show the potential phosphorylated Thr residues; the red lines show the potential phosphorylated Tyr residues. The light gray horizontal line indicates the threshold for modification potential. B: Predicted potential sites for *O*-GlcNAc modification on Ser and Thr residues in human CREB transcription factor. The green vertical lines show the *O*-GlcNAc modification potential of Ser/Thr residues and the light blue horizontal wavy line indicates the threshold for modification potential. C: Predicted potential sites for both *O*-GlcNAc modification and phosphorylation (Yin Yang sites). The positively predicted Yin Yang sites are shown with red asterisk at the top in human CREB transcription factor. The green vertical lines show the *O*-GlcNAc potential of Ser/Thr residue and the light blue horizontal wavy line indicates the threshold for modification potential. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

separate transcription activation domains. The KID domain (aa 101–160) that is induced by kinases and promotes transcription, and a glutamine-rich domain Q2 (also known as the constitutive active domain (CAD) (aa 160–280) that recruits and assembles a polymerase complex to the promoter, thus facilitating transcription [Ferreri et al., 1994; Kim et al., 2000]. These two domains work independently to regulate transcription of target genes. However, in some instances the two domains have been shown to work in a combined manner by stimulating various steps in initiation of transcription [Kim et al., 2000].

In this study, the potential phosphorylation, *O*-GlcNAc modification and their interplay sites (Yin Yang sites) were predicted in silico by using different computational tools (see Material and Methods Section). All the predicted phosphorylation sites (except for Ser260) in CREB have been experimentally verified in vivo or in vitro or proposed as putative sites by different groups (see Table II), but information regarding the functional changes induced by phosphorylation of CREB on proposed sites is limited. In forskolin-induced PC12 cells, phosphorylation of CREB on Ser133 by PKA followed by phosphorylation on Ser129 by glycogen

TABLE II. Experimental and In Silico Predicted Phosphorylation, *O*-Glycosylation, and Yin Yang Sites in Human CREB Transcription Factor

		Phosphorylation		*O*-GlcNAc modification	
Residue	Conservation status	Experimental verification[a]	Predicted by computational tool	Experimental verification	Predicted by computational tool
Serine					
5	:	–	–	–	Yes
12	.	–	–	–	Yes
40	:	–	–	–	Yes
48	:	–	–	–	Yes
49	:	–	–	–	Yes
76	*	–	–	–	Yes
88	* in mammals while DG in others	–	–	–	Yes
89	* in mammals while DG in others	Yes	Yes	–	–
98	* in mammals while DG in others	Yes	Yes	–	–
108	*	Yes	Yes	–	–
111	*	Yes	Yes	–	–
114	*	Yes	Yes	–	–
117	*	Yes	Yes	–	Yes
121	*	Yes	Yes	–	–
129	*	Yes	Yes	–	Yes
133[b]	*	Yes	Yes	–	–
142	*	Yes	–	–	–
143	*	Yes	–	–	Yes
156	*	Yes	Yes	–	–
178	*	–	–	–	Yes
260	*	–	Yes	Putative	Yes
271	*	–	–	–	Yes
340	*	–	–	–	Yes
Threonine					
18	:	–	Yes	–	Yes
35	*	–	–	–	Yes
47	:	–	–	–	Yes
54	*	–	–	–	Yes
85	*	–	–	–	Yes
104	*	–	–	–	Yes
119	*	–	–	–	Yes
160	:	–	Yes	–	Yes
166	:	–	–	–	Yes
167	*	–	Yes	–	Yes
169	*	–	–	–	Yes
215	:	–	Yes	–	–
220	*	–	–	–	Yes
227	*	–	Yes	–	Yes
228	:	–	–	–	Yes
256	*	–	–	Putative	–
259	:	–	–	Putative	Yes
261	*	–	–	Putative	Yes

The consensus sequence is marked by an asterisk, conserved substitution by a double dot, and semiconserved substitution by a single dot.
[a]Experimental determined phosphorylation sites are reviewed in Johannessen et al. [2004].
[b]Ser 133 is an in silico predicted false-negative Yin Yang site.

synthase kinase-3β (GSK-3β) increases transcription [Fiol et al., 1994]. Parathyroid hormone-stimulated osteoblastic cells require expression of *c-fos*, which is regulated by phosphorylation of CREB on Ser129 and 133 [Tyson et al., 2002]. Phosphorylation of Ser108, 110, and 114 which occurs in a cell cycle-dependent manner, increases CREB's activity [Johannessen et al., 2004], whereas phosphorylation on Ser142 by CAMKII prevent binding of CBP to CREB [Wu and McMurray, 2001]. Phosphorylation of Ser133 is the most extensively studied phosphorylation site in CREB. Mutational studies have shown that deletion or substitution of Ser133 in CREB inhibits its transcriptional activation in KID, but leaves basal activation unaltered [Gonzalez et al., 1989; Lee et al., 1990; Quinn, 1993]. But CREB phosphorylation on its own may not be sufficient to result in gene expression unless associated with the induction of L-LTP [Leutgeb et al., 2005]. Furthermore CREB phosphorylation is specific for CA1 as compared to CA3 and dentate gyrus in the hippocampus during long lasting LTP [Leutgeb et al., 2005]. Among the in silico predicted Yin Yang sites in human CREB, Ser133, is a predicted false negative Yin Yang site (Fig. 2, Table II). This suggests that OGT may play a role in the transcriptional regulation of CRE containing genes like *c-fos*. The *O*-GlcNAc modification is already known to occur in various proteins involved in gene expression such as the transcription factor Sp1 [Su et al., 1999; Yang et al., 2001] and the serum response factor SRF [Chalkley and Burlingame, 2003]. CREB is further recognized as an *O*-GlcNAc modified protein [Lamarre-Vincent and Hsieh-Wilson, 2003]. It was found that the putative *O*-GlcNAc sites are located in between aa 256–261. The positive in silico predicted Yin Yang sites are Ser117, 129, 260 and Thr18, 160, 227 (Table II). Ser260 resides in between aa 256–261 and is a likely candidate recognition site for OGT and could participate in CREB-regulated transcription. Furthermore the same authors have suggested that *O*-GlcNAc might

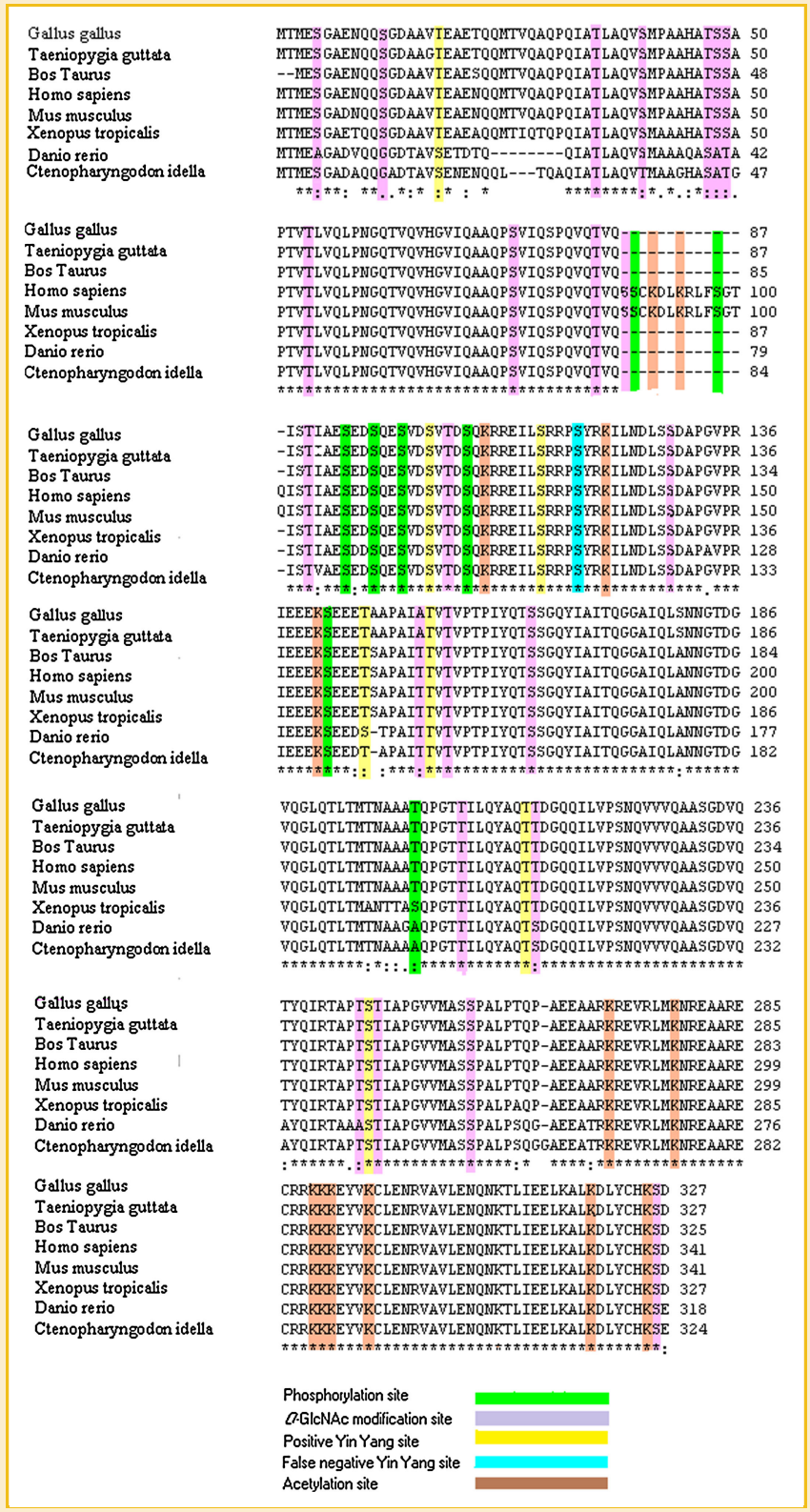

Fig. 2. Multiple alignments of different vertebrate sequences. The consensus sequence is marked by an asterisk, conserved substitution by a double dot, and semiconserved substitution by a single dot. The different sequences are ordered as in aligned results from ClustalW.

TABLE III. In Silico Predicted Acetylation Sites in Human CREB Transcription Factor

Peptide	Position
TVQSSCKDLKRLF	91
SSCKDLKRLFSGT	94
SVTDSQKRREILS	123
RRPSYRKILNDLS	136
PRIEEEKSEEETS	155
AEEAARKREVRLM	285
REVRLMKNREAAR	292
ARECRRKKKEYVK	303
RECRRKKKEYVKC	304
ECRRKKKEYVKCL	305
KKKEYVKCLENRV	309
EELKALKDLYCHK	333
KDLYCHKSD	339

play a role in the transcriptional function of CREB. In the transcription factor Sp1, *O*-GlcNAc modification disrupts the binding between Sp1 and the TATA-binding protein-associated factor II 110 ($TAF_{II}110$) [Yang et al., 2001], and it has been suggested that most often *O*-GlcNAc modified transcription factors are modified in their transcriptional activation domain [Jackson and Tjian, 1988; Comer and Hart, 1999]. CREB associates with the general transcription factor TFIID through one or more of the TATA-binding protein-associated factors (TAFs), TAF110 and $TAF_{II}130$ in the CAD domain [Felinski and Quinn, 1999; Kim et al., 2000], where the in silico predicted Yin Yang site Ser260 is located. Interaction of CREB with TAFs promotes recruitment and/or stabilization of TFIID binding to the promoter and leads to assembly of a pre-initiation polymerase complex. It was shown that *O*-GlcNAc modified CREB bound less to $TAF_{II}130$, consequently inhibiting CREB-mediated transcription [Lamarre-Vincent and Hsieh-Wilson, 2003]. This suggests an interplay between the phosphorylation and *O*-GlcNAc modifications occurs, which regulates the activity of CREB via its CAD domain. The Ser133 site located in the KID domain of CREB is an in silico predicted false negative Yin Yang site (Table II, Fig. 2) and this site can be phosphorylated by several kinases, depending on the stimulus [reviewed in Johannessen et al., 2004]. In PKA simulated cells, CREB becomes phosphorylated on Ser133 which becomes a recognition site for CBP/p300 [Parker et al., 1996]. Binding of CBP/p300 to CREB leads to acetylation of CREB on Lys91, 96, and 136 [Johannessen et al., 2004]. Acetylation is already known to play an important role in gene expression. In histone H3 acetylation increases transcription of immediate early genes (IE-genes) [Kaleem et al., 2008], and acetylation also enhances expression specific genes during the process of memory consolidation [Vecsey et al., 2007]. The in silico predicted acetylation sites are given in Table III and all three sites predicted by the PAIL server are potential acetylation sites.

As suggested by Kim et al. [2000], the two different transcriptional domains of CREB can work together to initiate gene transcription. These in silico results suggest that when CREB becomes phosphorylated on Ser133, CBP/p300 binds and consequently acetylates CREB. Furthermore CREB, via is CAD domain, recruits and assembles a complex consisting of the TATA-binding protein complex and RNA polymerase. While when CREB is *O*-GlcNAc modified, binding of CBP/p300 is inhibited, and in the CAD domain of CREB, *O*-GlcNAc modification prevents binding of $TAF_{II}130$, suggesting that the interplay between the different modification control CREB regulated transcription. The *O*-GlcNAc modification controls various aspects of transcription. The enzyme OGT recruits a complex (mSin3A-HDAC1) to promoters and induces gene repression by modifying different promoter-bound proteins such as the RNA polymerase [Yang et al., 2002]. OGT can also induce transcription by glycosylating the transcription factor STAT5 [Gewinner et al., 2004].

During learning, the synaptic plasticity (by alteration of synaptic protein number or structures) and gene expression occur simultaneously. Phosphorylation of CREB is important as a requirement for long-term memory consolidation. Phosphorylated CREB is also acetylated, which increases it transcriptional activity, whereas *O*-GlcNAc modified CREB represses transcription. These results indicate *O*-GlcNAc modified CREB prevents establishment of long-term memory in the hippocampus. In our earlier in silico work, we have shown that phosphorylated histone H3 is also acetylated and induces transcription of immediate-early genes *c-jun* and *c-fos*, but that *O*-GlcNAc modification of histone H3 represses transcription [Kaleem et al., 2008]. These investigations illustrate the complexity of CREB regulation by a combined activity between the different PTMs and consequently L-LTP in hippocampus.

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REFERENCES

Altschul SF, Madden TL, Scheffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSIBLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.

Balschun D, Wolfer DP, Gass P, Mantamadiotis T, Welzl H, Schutz G, Frey JU, Lipp HP. 2003. Does cAMP response element-binding protein have a pivotal role in hippocampal synaptic plasticity and hippocampus-dependent memory? J Neurosci 23:6304–6314.

Bernabeu R, Bevilaqua L, Ardenghi P, Bromberg E, Schmitz P, Bianchin M, Izquierdo I, Medina JH. 1997. Involvement of hippocampal cAMP/cAMP-dependent protein kinase signaling pathways in a late memory consolidation phase of aversively motivated learning in rats. Proc Natl Acad Sci USA 94: 7041–7046.

Blom N, Gammeltoft S, Brunak S. 1999. Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol 294:1351–1362.

Boeckmann B, Bairoch A, Apweiler R, Blatter MC, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O'Donovan C, Phan I, Pilbout S, Schneider M. 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res 31:365–370.

Boehm J, Malinow R. 2005. AMPA receptor phosphorylation during synaptic plasticity. Biochem Soc Trans 33:1354–1356.

Broadbent NJ, Squire LR, Clark RE. 2004. Spatial memory, recognition memory, and the hippocampus. Proc Natl Acad Sci USA 101:14515–14520.

Cardinaux J-E, Notis JC, Zhang Q, Vo N, Craig JC, Fass DM, Brennan RG, Goodman RH. 2000. Recruitment of CREB binding protein is sufficient for CREB-mediated gene activation. Mol Cell Biol 20:1546–1552.

Chalkley RJ, Burlingame AL. 2003. Identification of novel sites of O-GlcNAc modification of serum response factor using Q-TOF mass spectrometry. Mol Cell Proteomics 2:182–190.

Cole RN, Hart GW. 2001. Cytosolic O-glycosylation is abundant in nerve terminals. J Neurochem 79:1080–1089.

Comer FI, Hart GW. 1999. O-GlcNAc and the control of gene expression. Biochim Biophys Acta 1473:161–171.

Comer FI, Hart GW. 2000. O-Glycosylation of nuclear and cytosolic proteins: Dynamic interplay between O-GlcNAc and O-Phosphate. J Biol Chem 275: 29179–29182.

Doyle KP, Simon RP, Stenzel-Poore MP. 2008. Mechanisms of ischemic brain damage. Neuropharmacology 55:310–318.

Felinski EA, Quinn PG. 1999. The CREB constitutive activation domain interacts with TATA-binding protein-associated factor 110 (TAF110) through specific hydrophobic residues in one of the three subdomains rquired for both activation and TAF110 binding. J Biol Chem 274:11672–11678.

Ferreri K, Gill G, Montminy M. 1994. The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. Proc Natl Acad Sci USA 91:1210–1213.

Fiol CJ, Williams JS, Chou CH, Wang QM, Roach PJ, Andrisani OM. 1994. A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. J Biol Chem 269:32187–32193.

Gewinner C, Hart G, Zachara N, Cole R, Beisenherz-Huss C, Groner B. 2004. The coactivator of transcriptionCREB-binding protein interacts preferentially with the glycosylated form of Stat5. J Biol Chem 279: 3563–3572.

Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, Greenberg ME. 1993. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260: 238–241.

Gonzalez GA, Yamamoto KK, Fischer WH, Karr D, Menzel P, Biggs WI, Vale WW, Montminy MR. 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337: 749–752.

Gupta R, Brunak S. 2002. Prediction of glycosylation across the human proteome and the correlation to protein function. Pac Symp Biocomput 7:310–322.

Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR. 1996. Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 16:973–982.

Jackson SP, Tjian R. 1988. O-glycosylation of eukaryotic transcription factors: Implications for mechanisms of transcriptional regulation. Cell 55:125–133.

Jin K, Peel AL, Mao XO, Xie L, Cottrell BA, Henshall DC, Greenberg DA. 2004. Increased hippocampal neurogenesis in Alzheimer's disease. Proc Natl Acad Sci USA 101:343–347.

Johannessen M, Delghandi MP, Moens U. 2004. What turns CREB on? Cell Signal 16:1211–1227.

Kaleem A, Hoessli DC, Ahmad I, Walker-Nasir E, Nasim A, Shakoori AR, Nasir-ud-Din. 2008. Immediate-early gene regulation by interplay between different post-translational modifications on human histone H3. J Cell Biochem 103:835–851.

Kandel ER. 2001. The molecular biology of memory storage: A dialog between genes and synapses. Science 294:1030–1038.

Kim J, Lu J, Quinn PG. 2000. Distinct cAMP response element-binding protein (CREB) domains stimulate different steps in a concerted mechanism of transcription activation. Proc Natl Acad Sci USA 97:11292–11296.

Korzus E. 2003. The relation of transcription to memory formation. Acta Biochim Pol 50:775–782.

Lamarre-Vincent N, Hsieh-Wilson LC. 2003. Dynamic glycosylation of the transcription factor CREB: A potential role in gene regulation. J Am Chem Soc 125:6612–6613.

Lee CQ, Yun YD, Hoeffler JP, Habener JF. 1990. Cyclic-AMP-responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. EMBO J 9:4455–4465.

Lee H-Y, Chaudhary J, Walsh GL, Hong WK, Kurie JM. 1998. Suppression of c-Fos gene transcription with malignant transformation of human bronchial epithelial cells. Oncogene 16:3039–3046.

Leutgeb JK, Frey JU, Behnisch T. 2005. Single cell analysis of activity-dependent cyclic amp-responsive element-binding protein phosphorylation during long-lasting long-term potentiation in area CA1 of mature rat hippocampal–organotypic cultures. Neuroscience 131:601–610.

Lipton P. 1999. Ischemic cell death in brain neurons. Physiol Rev 79:1431–1568.

Lonze BE, Ginty DD. 2002. Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605–623.

Love DC, Hanover JA. 2005. The hexosamine signaling pathway: Deciphering the "O-GlcNAc code". Science STKE 312:re13.

Lu Q, Hutchins AE, Doyle CM, Lundblad JR, Kwok RPS. 2003. Acetylation of CREB by CBP enhances CREB-dependent transcription. J Biol Chem 278: 15727–15734.

Mizuno M, Yamada K, Maekawa N, Saito K, Seishima M, Nabeshima T. 2002. CREB phosphorylation as a molecular marker of memory processing in the hippocampus for spatial learning. Behav Brain Res 133:135–141.

Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci USA 83:6682–6686.

O'Connell C, Gallagher HC, O'Malley A, Bourke M, Regan CM. 2000. CREB Phosphorylation coincides with transient synapse formation in the rat hippocampal dentate gyrus following avoidance learning. Neural Plast 7: 279–289.

Parker D, Ferreri K, Nakajima T, Lamorte VJ, Evans R, Koerber SC, Hoeger C, Montminy MR. 1996. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. Mol Cell Biol 16:694–703.

Quinn PG. 1993. Distinct activation domains within cAMP response element-binding protein (CREB) mediate basal and cAMP-stimulated transcription. J Biol Chem 268:16999–17009.

Routtenberg A, Rekart JL. 2005. Post-translational protein modification as the substrate for long-lasting memory. Trends Neurosci 28:12–19.

Schaub C, Uebachs M, Beck H. 2007. Diminished response of CA1 neurons to antiepileptic drugs in chronic epilepsy. Epilepsia 48:1339–1350.

Sekino Y, Obata K, Tanifuji M, Mizuno M, Murayama J. 1997. Delayed signal propagation via CA2 in rat hippocampal slices revealed by optical recording. J Neurophys 78:1662–1668.

Sennels L, Salek M, Lomas L, Boschetti E, Righetti PG, Rappsilber J. 2007. Proteomic analysis of human blood serum using peptide library beads. J Proteom Res 6:4055–4062.

Su K, Roos MD, Yang X, Han I, Paterson AJ, Kudlow JE. 1999. An N-terminal region of Sp1 targets its proteasome-dependent degradation in vitro. J Biol Chem 274:15194–15202.

Sunyer B, Diao W, Lubec G. 2008. The role of post-translational modifications for learning and memory formation. Electrop 29:2593–2602.

Thompson JD, Higgins DG, Gibson TJ. 1994. ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Tyson DR, Swarthout JT, Jefcoat SC, Partridge NC. 2002. PTH induction of transcriptional activity of the cAMP response element-binding protein requires the serine 129 site and glycogen synthase kinase-3 activity, but not casein kinase II sites. Endocrinology 143:674–682.

Vecsey CG, Hawk JD, Lattal KM, Stein JM, Fabian SA, Attner MA, Cabrera SM, McDonough CB, Brindle PK, Abel T, Wood MA. 2007. Histone deace-

tylase inhibitors enhance memory and synaptic plasticity via CREB: CBP-dependent transcriptional activation. J Neurosci 27:6128–6140.

Vo N, Goodman RH. 2001. CREB-binding protein and p300 in transcriptional regulation. J Biol Chem 276:13505–13508.

Walker WH, Sanborn BM, Habener JF. 1994. An isoform of transcription factor CREM expressed during spermatogenesis lacks the phosphorylation domain and represses cAMP-induced transcription. Proc Natl Acad Sci USA 91:12423–12427.

AU>Hart GW. 2003. O-GlcNAc: A regulatory post-translational modification. Biochem Biophys Res Commun 302:435–441.

Wu X, McMurray CT. 2001. Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein. J Biol Chem 276: 1735–1741.

Yang X, Su K, Roos MD, Chang Q, Paterson AJ, Kudlow JE. 2001. O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. Proc Natl Acad Sci USA 98:6611–6616.

Yang X, Zhang F, Kudlow JE. 2002. Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: Coupling protein O-GlcNAcylation to transcriptional repression. Cell 110:69–80.

Zhang X, Odom DT, Koo S-H, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M. 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA 102:4459–4464.

Zhu JJ. 2003. Mechanisms of synaptic plasticity: From membrane to intracellular AMPAR trafficking. Mol Interv 3:15–18.