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New Phenomenon

MBD 4—a potential substrate for protein kinase X

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Human protein kinase X (PrKX) is an X chromosome-encoded cAMP-dependent protein kinase. PrKX has 50.2%, 50.8%, and 44.83% identity with the catalytic, C-subunit of PKAα, PKAβ, and PKAγ, respectively [1]. PrKX shares some biochemical characteristics with PKA. Both kinases catalyze phosphorylation of histone H1 and the PKA synthetic septapeptide substrate, referred to as Kemptide (LRRASLG), *in vitro*. However, the specific activities of PrKX phosphorylation of histone H1 and Kemptide are significantly lower than that of PKA [2,3]. The RII regulatory subunit of PKA is an excellent substrate of PrKX even in the absence of cAMP. The catalytic activity can be specifically inhibited by PKI, a heat-stable physiological polypeptide inhibitor of PKA [2]. PrKX stably associates with RI but not with RII *in vivo* [2,4]. Dibutyryl-cAMP, a cell-permeable analog of cAMP, induces the translocation of PrKX from cytoplasm into the nucleus [2]. Transient expression assays have shown that PrKX is capable of activating cAMP-response-element-binding protein-dependent transcription similar to PKA [5].

Despite the reported similarities between PrKX and PKA, other studies have indicated that there are functional differences between the two kinases. PrKX involvement in granulocyte/macrophage lineage differentiation has been suggested [6]. Smad6 is a proven PrKX substrate mediating the differentiation processes [7]. PrKX appears to activate branching morphogenesis and cellular migration of kidney cells [5]. Another study implicates Pkare, the mouse homolog of PrKX is involved in neuronal development [8]. None of the above functions are observed for PKA, which suggests that the substrate preferences of the two kinases differ. Although studies have revealed important functions of PrKX, few substrates have been identified to date.

By using an yeast two-hybrid system, we identified that MBD4 may be a new substrate for PrKX. The entire human PrKX open reading frame was ligated in-frame to the carboxy-terminus of the GAL_4 DNA-binding domain supplied in plasmid $pGBKT_7$. The resulting construct, $pGBKT_7$-hPrKX, provided the 'bait' for the interaction trap screen. The bait-plasmid was transformed into *Saccharomyces cerevisiae* strain AH109 (phenotypically ADE^-, HIS^-, Leu^-, Trp^-) and tested for transcriptional activation. Yeast mating was performed with strain AH109 harboring bait plasmid and Y187 pre-transformed with a human bone marrow cDNA library constructed with a pACT2 vector. The mating mixture was plated onto SD-Leu-Trp-His with 20 mM 3-AT for preliminary screening. Between 3 and 8 days after plating, colonies grew and were isolated. Plasmid DNA was extracted by using an yeast plasmid DNA isolation kit. To isolate individual plasmid, the plasmid preparations were then separately transformed into AH109 harboring bait-plasmid or empty $pGBKT_7$ plasmid and plated onto SD-Leu-Trp-His-ADE with the chromogenic substrate X-α-Gal allowing for color selection indicating protein–protein interaction. Within 8 days, blue colonies grew which were isolated and the plasmid DNA recovered. The DNA sequence of the cDNA inserts was determined.

In the yeast two-hybrid screening, 20 library plasmids from robust blue colonies were sequenced out of the 42 clones isolated. Three cDNA sequences corresponded to the human methyl-CpG binding domain protein 4, hMBD4 (GeneBank accession No. BC011752). To confirm the result, yeast AH109 was transformed with the following: empty AD vector and bait, or empty BD vector and AD-hMBD4 construct, or AD-hMBD4 vector with bait. It was found that only MBD4 fused with AD vector cotransformed with bait could activate Ade + LacZ reporter (**Fig. 1**).

MBD4 is a mammalian DNA glycosylase that contains both an N-terminal methyl-CpG binding domain (MBD) and a C-terminal DNA glycosylase domain. MBD specifically binds methylated DNA and glycosylase domain removes thymine or uracil base from thymine or uracil mispaired with guanine [9]. The MBD4 protein was previously described as a phosphoprotein. Evidences have also implicated the relevance of phosphorylation with

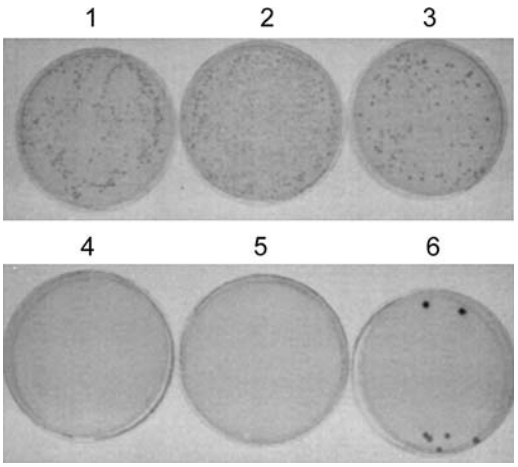

Figure 1 Yeast two-hybrid screen Yeast strain AH109 was transformed with BD-hPrKX plus empty AD plasmids, AD-hMBD4 fusion plus empty BD plasmids, or BD-hPrKX plus AD-hMBD4 and then spread on SD-Leu-Trp (upper panel 1, 2, and 3 respectively) and SD-Leu-Trp-His-Ade + X-α-Gal (lower panels 4–6). While all clones grew under non-selective conditions, only yeast transformed with both BD-hPrKX and AD-MBD4 grew under selective conditions.

MBD4 function [10]. However, the kinases involved in the phosphorylation have not been identified. The interaction between PrKX and MBD4 is a novel finding that has not been reported. The interaction suggests that MBD4 is a potential substrate for PrKX. More subsequent work needs to be done to confirm the preliminary result.

Future work will concentrate on answering the following questions: (i) Whether PrKX interacts with MBD4 in *in vitro* and *in vivo*. These can be done by GST-pull-down and coimmunoprecipition experiments. (ii) Whether PrKX phosphorylates MBD4 *in vitro* and *in vivo*. *In vitro* kinase assay and intracellular overexpression or knockdown of PrKX protein combined with radioactive metabolic phosphate labeling can be performed. (iii) Whether the phosphoryaltion of MBD4 modulates its physiological activities (including DNA binding and/or DNA glycosylase activities). Gel shift and glycosylase activity assay can be employed to study the effects of phosphoryaltion on MBD4 activities. (iv) Whether MBD4 is a specific substrate for PrKX or it is a common substrate shared by PrKX and PKA. This can be done by performing the same experiments as above. These works will clarify the substrate specificity and the physiological implications of our finding.

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