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# Development of a Protein Chip to Measure PKCβ Activity

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**Abstract** Phosphorylation of proteins by kinases plays an important role in regulating cellular processes including melanin production in the skin cells. Protein kinase C β (PKCβ) is known to be involved in phosphorylating tyrosinase, the key enzyme of melanin production, regulating the skin pigmentation process. In melanogenesis, PKCβ activates the tyrosinase by phosphorylation of its two serine residues. In this study, phosphorylation activity by PKCβ was monitored on a protein chip for the screening of depigmenting agents. As a tyrosinase mimic, 11 or 30 amino acids of the C-terminal of tyrosinase was fused with maltose-binding protein (MBP). After immobilizing the MBP-fused PKCβ substrate peptide on epoxy-treated slide surface, PKCβ reaction mix was applied over the immobilized MBP-fused PKCβ substrate peptide. Phosphorylation was detected with anti-phosphoSer/Thr antibodies, followed by fluorescence-labeled second antibodies. Phosphorylation of MBP-30aa was observed on a protein chip, and this phosphorylation was inhibited by the PKC inhibitor (GF109203X). These results indicate the potential of PKCβ protein chip as a high-throughput screening tool in the screening of depigmenting agents.

**Keywords** PKCβ · Tyrosinase · Protein chip · HTS · Depigmenting agents

**Abbreviations**

| | |
|---|---|
| PKC | Protein kinase C |
| MBP | Maltose-binding protein |
| TMP | Tyrosinase mimic peptide |
| BSA | Bovine serum albumin |

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## Introduction

Excessive exposure of skin to sunlight results in skin hyperpigmentation. Some skin disorders include melasma, post-inflammatory hyperpigmentation, and lentigo snilis [1]. The hyper-pigmentation disorders can be medically treated with skin-lightening agents and/or treated with cosmeceuticals, defined as cosmetic ingredients which have a curative potential. Although many ingredients have been developed and are currently used in dermatology as well as in the cosmetic industry, safer, more effective, and less irritating lightening agents are still required [1].

Melanin production is principally responsible for skin color and plays an important role in the prevention of sun-induced skin injury [2]. Melanin is produced in melanocytes, neural crest-derived cells residing at the basal layer of the epidermis; tyrosine and L-dihydroxyphenylalanin (L-dopa) serve as precursors for this complex biopolymer [2,3]. These precursor amino acids are oxidized by a rate-limiting enzyme tyrosinase [4,5], and subsequent reactions involving tyrosinase and tyrosinase-related proteins result in the deposition of melanin pigment in specialized organelles termed melanosomes [2].

The protein kinase C (PKC)-dependent pathway has emerged as a second intracellular signaling pathway regulating melanogenesis. It was first observed that addition of diacylglycerol, the endogenous activator of PKC, to cultured human melanocytes caused a rapid three to fourfold increase in total melanin content, and this increase was blocked by a PKC inhibitor [6]. In cultured pigment cells, depletion of PKC reduced the melanin content dramatically [7–9] and completely blocked α-MSH-induced melanogenesis. Moreover, it has been reported that the β-isoform of PKC was specifically involved in melanogenesis and that the specific role of PKCβ was to phosphorylate (activate) tyrosinase [10]. The cytoplasmic domain of tyrosinase contains two serine residues at amino acid positions 505 and 509 [11], and both serine residues are phosphorylated by PKCβ, resulting in the activation of tyrosinase [12].

Since many reports demonstrated that PKCβ can be a potential target of development of depigmenting agents, we aimed to construct a protein chip to measure a PKCβ activity.

In this study, we developed a protein chip by immobilizing a PKCβ substrate peptide sequence fused to maltose-binding protein (MBP) onto an epoxy-treated glass plate. Phosphorylation of the protein chip was monitored and its potential use as a screening tool was investigated. The results suggest that this protein chip can be used as a screening tool for depigmenting agents from numerous natural compounds as well as from chemical libraries.

## Materials and Methods

### Materials

A protein kinase CβII was purchased from Calbiochem. Isopropyl-D-thiogalactopyranoside (IPTG), tyrosinase mimic peptide (TMP), histone, and bisindolylmaleimide I were obtained from Sigma. Mouse monoclonal phospho-Ser/Thr antibody and anti-mouse IgG second antibody were purchased from Zymed and Santa Cruz, respectively.

### Production of MBP Fusion Peptide and MBP-c2X

To construct a MBP-fused human tyrosinase peptide expression vector, a polymerase chain reaction (PCR) was performed using each primer set as follows: TYR-30aa 5′-GGC CCA

TGG GAC GAC GAC GAC AAG TGT CGT CAC AAG AGA AAG CAG CTT-3′ (forward) 5′-GGC CTC GAG TTA TAA ATG GCT CTG ATA CAA ACT-3′ (reverse); TYR-11aa 5′-GGC CCA TGG GAC GAC GAC GAC AAG GAG GAT TAC CAC AGT-3′ (forward) 5′-GGC CTC GAG TTA TAA ATG GCT CTG ATA-3′ (reverse). After the PCR reaction, the PCR product was purified and digested with the restriction enzymes *Nde*I and *Xho*I. The resulting DNA fragment was ligated into the *Nde*I and *Xho*I sites of the pET21a vector with a DNA fragment encoding 30 amino acids or 11 amino acids of human tyrosinase. The transformed cells, *Escherichia coli* BL21/pET21aMBP30 and *E. coli* BL21/pET21aMBP11, were cultured in Luria–Bertani media and allowed to grow at 30 °C to an OD of 0.6 before 1 mM IPTG was added. After IPTG induction, the cells were cultured for an additional 4 h followed by centrifugation at 5,000 rpm for 5 min. The resulting pellets were suspended in 50 mM Tris–HCl buffer (pH 8.0) and broken up by sonication for 5 min. After sonication, the cell lysate was again centrifuged at 5,000 rpm for 5 min. MBP-30aa or MBP-11aa was purified by MBP Affinity Chromatography (Bioprogen, Korea) using elution buffer (10 mM maltose, 20 mM Tris–HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4). As a control, MBP-c2X was produced from the vector pMAL-c2X (New England Biolabs Inc.) which contains MBP.

## Construction of the PKCβ Protein Chip

First, the protein chip containing MBP-30aa was developed as previously described [13]. On epoxy-treated glass slides (CEL associates, Inc.), well stickers were attached to make a well chip. Recombinant MBP-30aa protein was immobilized on the epoxy-treated slide surface by immersing the MBP-30aa (6 μM) overnight, followed by washing with PBS-T (0.5% Tween 20 in PBS) for 20 min and drying in $N_2$ stream. Unbound epoxy residues were blocked by immersion in 3% bovine serum albumin (BSA) solution for 1.5 h, treated with PKCβ reaction mix at 30 °C, washed, and immersed in antibodies (anti-phosphoSer/Thr antibody and Cy5-labeled secondary antibody). The fluorescence intensity was measured by fluorescence scanner.

## Phosphorylation of MBP-30aa in a Well Chip and Detection by Fluorescence

To measure the kinase activity of PKCβ in well chip, fabricated well chip was immersed in 0.5 mg/ml MBP-30aa solution, washed with PBS-T, dried in $N_2$ stream, and blocked with 3% BSA as described above. Next, 100 μl of PKCβ reaction mix (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 μg/ml phosphatidylserine, 20 μg/ml diacylglycerol, 0.03% triton X-100, and 0.01 mM ATP) including 15.3 nM PKCβ enzyme was spotted in well chip in tetraplicates and incubated at 30 °C for 1 h. After washing with PBS-T, well chip was immersed in a solution of anti-phospho-Ser/Thr antibody and incubated at 30 °C for 1 h. For the detection of binding, well chip was incubated with Cy5-labeled secondary antibody at 30 °C for 30 min [14]. Fluorescence intensity was measured by a fluorescence scanner GenePix 4100A (Axon) and GenePix 4.1 software program.

To examine the effect of two well-known PKCβ inhibitors, various concentrations of PKCβ inhibitors were added to the PKCβ reaction mix and were spotted in well chip. Other processes were the same as above.

To confirm the phosphorylation of PKCβ substrates (histone, MBP-30aa, and MBP-10aa) in a solution, PKCβ assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The reaction was performed in a microtube including 15 μl of PKCβ reaction mix containing 0.2 μCi of [$^{32}$P] ATP and 5 μl of one of

the PKCβ substrates tested (4 mg/ml of histone, MBP-c2X, MBP-11aa, MBP-30aa, or BSA). After 5-min incubation at 30 °C, the reaction was stopped by adding a half volume of 3X Laemli buffer with subsequent boiling for 2 min. The stopped mixture was run in 10% SDS-PAGE gel, the portion of gel containing unreacted free ATP was removed, and the remaining gel was stained using Coomassie Brilliant Blue and dried. The $^{32}$P radioactivity in the stained PKCβ protein band was visualized by autoradiography.

### Calculation of $Z'$ Factor

The $Z'$ factor is defined in terms of four parameters: the means and standard deviations of both the positive (p) and negative (n) controls ($\mu_p$, $\delta_p$ and $\mu_n$, $\delta_n$) [15]. Given these values, the $Z'$ factor is defined as:

$$Z'\text{factor} = 1 - \frac{3 \times \left(\delta_p + \delta_n\right)}{\left|\mu_p - \mu_n\right|}$$

The $Z'$ factor, estimated from the sample means and sample standard deviations, was calculated from each of 30 samples based on fluorescence readings in 30 wells with substrates and 32 wells with BSA.

### Statistics

Negative BSA control signals were used to determine background signals for each PKCβ assay. For each spot, the mean background value was subtracted from the signal for spots in each PKCβ assay. Data were analyzed using Plot ANY mathematical function of Sigma plot, version 9.0, and SigmaStat 3.1, and the error bars in each figure represent SEM of tetraplicates in more than three experiments.

## Results

### Selection of PKCβ Substrates

The protein chip containing PKCβ was developed as illustrated in Fig. 1. To determine whether MBP-30aa and MBP-11aa can be phosphorylated by PKCβ, phosphorylation of these substrates was confirmed using [$^{32}$-γ-P]-ATP. Substrates were added to the PKCβ reaction mix, incubated for 5 min, and subjected to SDS-PAGE. Figure 2 demonstrates that MBP-30 is a suitable substrate for our PKCβ assay system. To confirm the substrate potential of MBP-30, a PKCβ assay using the protein chip was performed. Phosphorylation of MBP-30 was detected by an antibody-based fluorescence measurement composed of phospho-Ser antibody and a Cy5-labeled secondary antibody. The PKCβ protein chip assay of several substrates showed phosphorylation of MBP-30aa as well as histone, but not of MBP-11aa or MBP-c2X (Fig. 3). These results are consistent with Fig. 2.

### Optimization of Concentrations of PKCβ, MBP-30, and ATP

For a reliable and reproducible experiment, we have confirmed the optimum concentration of the PKCβ. The kinase reactions were carried out in microtubes with a volume of 100 μl reaction buffer in the presence of different concentrations of PKCβ as indicated in Fig. 4.

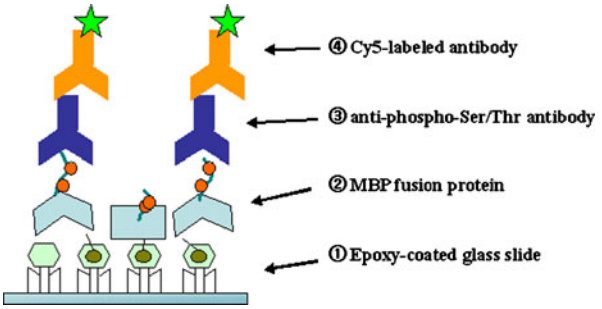


**Fig. 1** Schematic illustration of the process of PKCβ protein chip construction. The fusion peptide (MBP-30aa) was immobilized on an epoxy-coated glass plate. Anti-phospho-Ser/Thr antibody and a Cy5-labeled secondary antibody were arrayed serially after kinase reaction

Fluorescence intensity increased in proportion to the concentrations of PKCβ, suggesting that phosphorylation of substrates (histone or MBP-30aa) was due to PKCβ (Fig. 4a, b). However, high dose in PKCβ will increase the background signal. Therefore, to obtain clear results, applying a low concentration of PKCβ, such as 15.3 nM, was found to be better than a high concentration of PKCβ (76.7 or 153.4 nM; Fig. 4a). To confirm that the detected phosphorylation was due to the phosphorylation of substrate, we varied the concentrations of MBP-30aa. When the concentrations of MBP-30aa were varied, fluorescence intensity increased in proportion to an increase of MBP-30aa (Fig. 5), suggesting that increased fluorescence was due to the phosphorylation of MBP-30aa. The

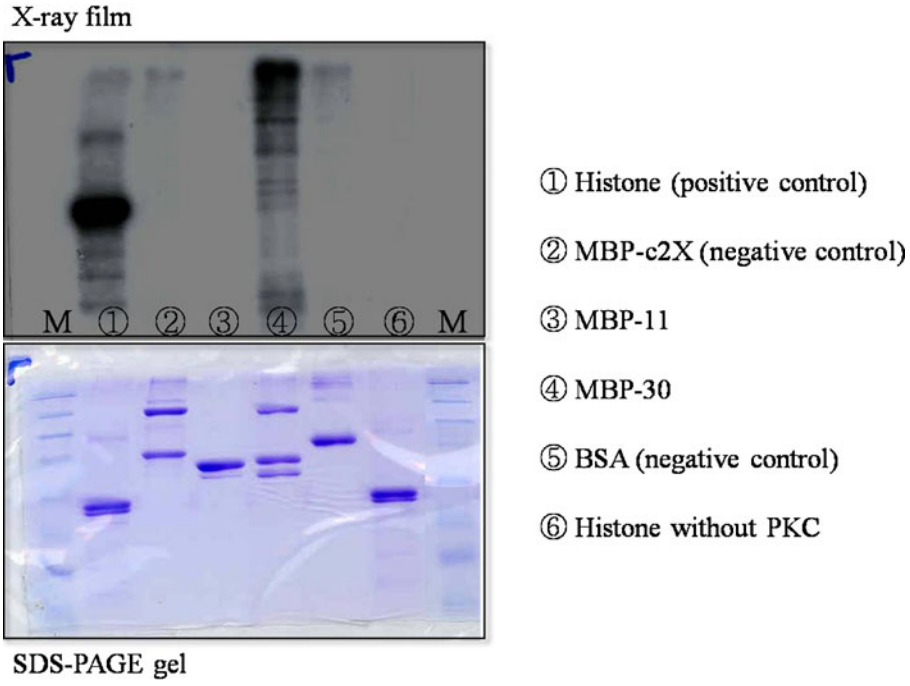


**Fig. 2** Selection of PKCβ substrates. The assays were carried out as described in "Materials and Methods." Samples were subjected to SDS-PAGE and Coomassie Brilliant Blue staining and autoradiography

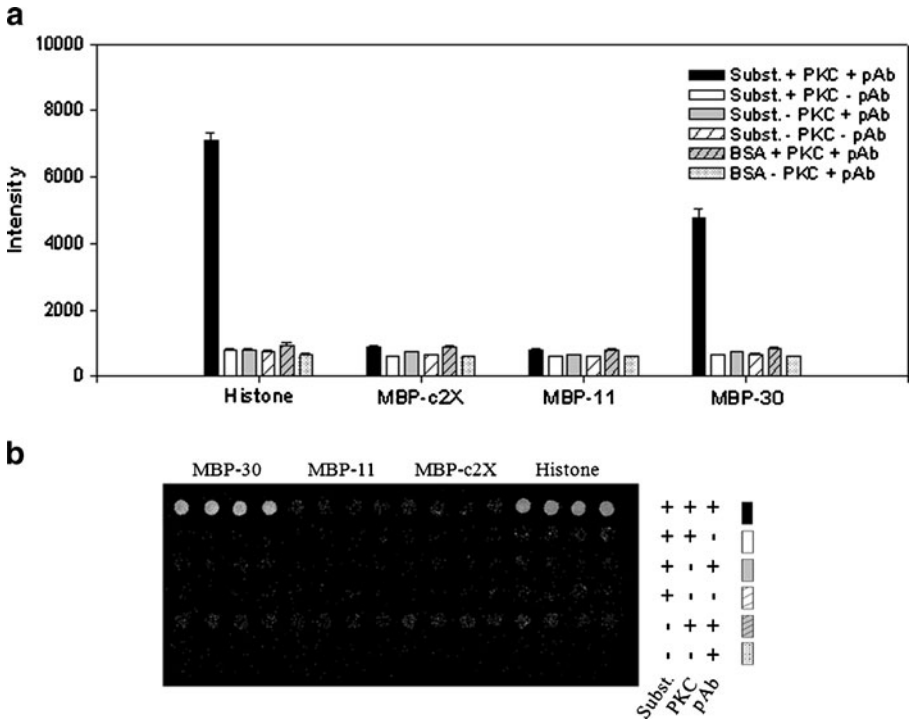


**Fig. 3** Selection of PKCβ substrates. The assays were carried out as illustrated in Fig. 1. Reaction mix was spotted in one of the four substrate-immobilized well chips (histone, MBP-c2X, MBP-11aa, or MBP-30aa) and incubated at 30 °C for 1 h. The slides were incubated with a series of anti-phospho-Ser/Thr antibody and a Cy5-labeled secondary antibody, washed, dried, scanned, and the fluorescence intensity of the spots was measured. The *error bars* represent the difference of tetraplicates in the experiment

$K_m$ value for MBP-30aa was determined ($K_m$=1 μM). When we varied the concentrations of ATP, another substrate of PKCβ, the fluorescence intensity was proportional to the increase of ATP, suggesting that the detected phosphorylation was due to the phosphorylation by PKCβ. The $K_m$ value for ATP was determined ($K_m$=0.1 μM). This kinetic model is fit for the Michaelis–Menten model (Fig. 6).

## Calculation of $Z'$ Factor

A recently developed simple statistical parameter ($Z'$ factor) is a better measure of high-throughput screening (HTS) assay quality than S/B (signal-to-background) [15]. Thus, we used a formula from Zhang et al. [15] to calculate the $Z'$ factor from our data. The $Z'_{30/32}$ factor was calculated based on fluorescence reading measured in the presence of PKCβ in 30 wells with MBP-30aa (signal) and 32 wells with BSA control (background). We determined $Z'_{30/32}$=0.704 from our data as being the "excellent assay" range [15]. These results show that this method was sensitive and could analyze multiple reagents simultaneously [16].

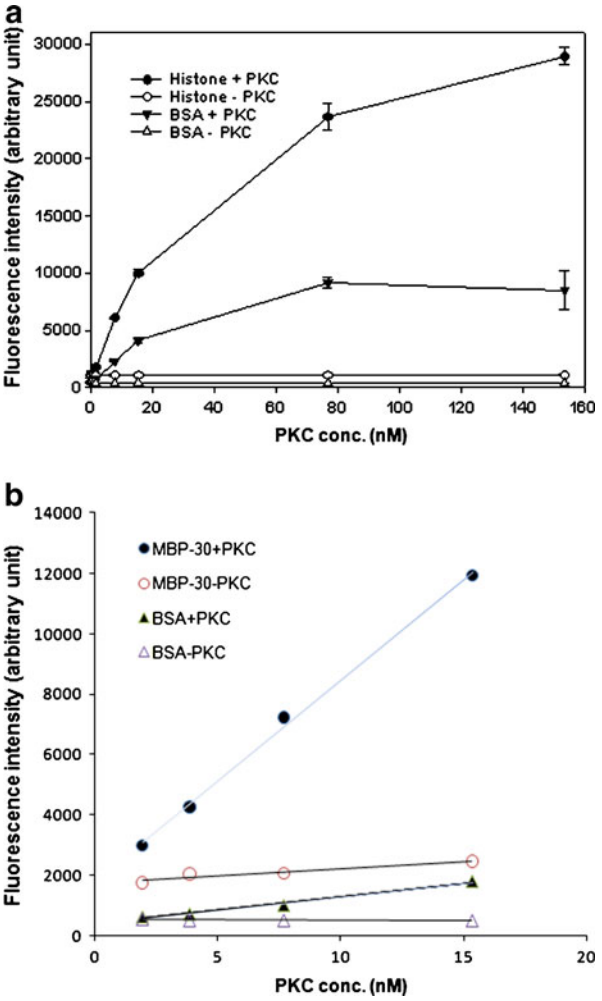


**Fig. 4** Effect of increasing concentrations of PKCβ on the phosphorylation of histone or MBP-30aa. Reaction mix including various concentrations of PKCβ was spotted in a histone (**a**) or an MBP-30aa (**b**) immobilized well chip and incubated at 30 °C for 1 h. The slides were incubated with a series of anti-phospho-Ser/Thr antibody and a Cy5-labeled secondary antibody, washed, dried, scanned, and the fluorescence intensity of the spots was measured. The fluorescence intensity is directly proportional to the phosphorylated substrate concentration, and the concentration-dependent kinase activity can be determined using an anti-phospho-Ser/Thr antibody and Cy5-labeled secondary antibody. The *error bars* represent the difference of tetraplicates in the experiment

Inhibition of PKCβ Activity by TMP and Bisindolylmaleimide I

To confirm the effectiveness as an inhibitor screening tool, two reported inhibitors, TMP and bisindolylmaleimide I, were used. As shown in Fig. 7a, b, TMP and bisindolylmaleimide I showed inhibitory effects against PKCβ in the protein chip, consistent with previous reports [17]. Figure 7b shows a representative inhibition curve of PKCβ.

## Discussion

Protein chip technology provides a new and useful tool for high-throughput screening of drugs and cosmetic ingredients because of its high performance and low sample

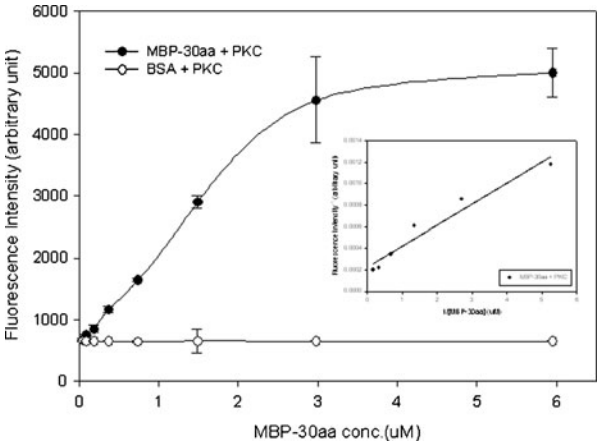


**Fig. 5** Effect of increasing concentrations of MBP-30aa on phosphorylation of MBP-30aa. Reaction mix was spotted in an MBP-30aa-immobilized well chip and incubated at 30 °C for 1 h. The slides were incubated with a series of anti-phospho-Ser/Thr antibody and a Cy5-labeled secondary antibody, washed, dried, scanned, and the fluorescence intensity of the spots was measured. The *error bars* represent the difference of tetraplicates in the experiment

consumption [18,19]. We developed a protein chip which can measure PKCβ activity using antibody-based fluorescence. PKCβ plays an important role in melanogenesis. PKCβ has been reported to phosphorylate tyrosinase, which is a key enzyme involved in melanogenesis. Therefore, we aimed to construct a tool that can be used in HTS for the development of depigmenting agents.

In the present study, we have evaluated several candidates for their potential as substrates of PKCβ in our protein chip assay system as well as in a conventional kinase assay system. As shown in Figs. 2 and 3, MBP-30aa is a suitable substrate of PKCβ involved in melanogenesis.

Next, we optimized the concentration of PKCβ, MBP-30aa (the substrate), and ATP. When we increased the dose in PKCβ, MBP-30aa, or ATP, fluorescence intensity increased correspondingly, suggesting the sensitivity of the protein chip (Fig. 4, 5, and 6).

As shown in Fig. 4a, a high dose of PKCβ also increased the background signal. PKCβ has many serine residues for autophosphorylation. There are possibilities that phosphor-

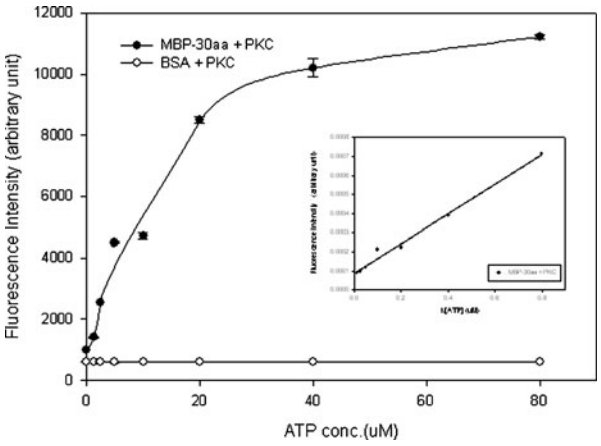


**Fig. 6** Effect of increasing concentrations of ATP on phosphorylation of MBP-30aa. Reaction mix including various concentrations of ATP was spotted in an MBP-30aa- immobilized well chip and incubated at 30 °C for 1 h. The slides were incubated with a series of anti-phospho-Ser/Thr antibody and a Cy5-labeled secondary antibody, washed, dried, scanned, and the fluorescence intensity of the spots was measured. The *error bars* represent the difference of tetraplicates in the experiment

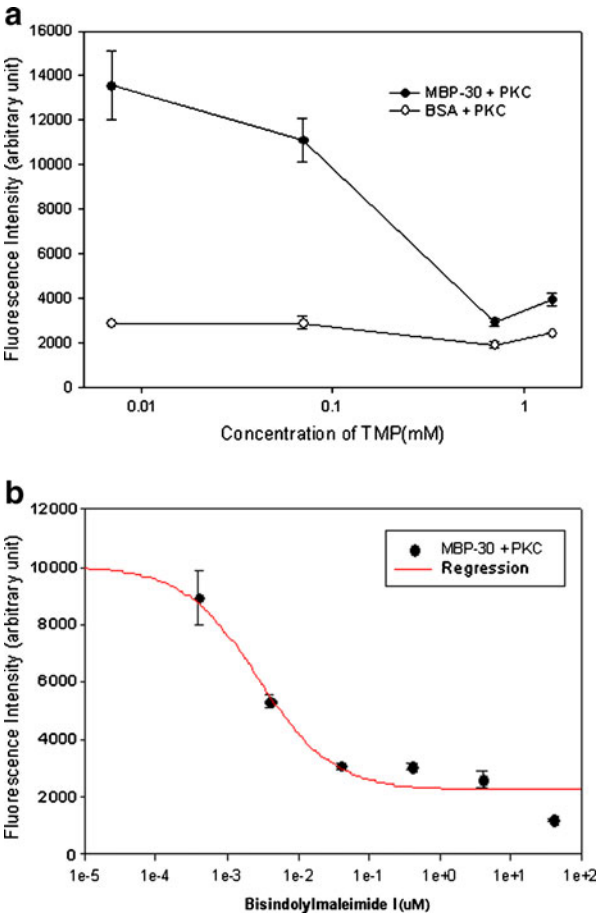


**Fig. 7** Inhibition of the PKC kinase activity by TMP and bisindolylmaleimide I. Reaction mix including different concentrations of TMP (**a**) or bisindolylmaleimide I (**b**) was spotted in an MBP-30aa-immobilized well chip and incubated at 30 °C for 1 h. The slides were incubated with a series of anti-phospho-Ser/Thr antibody and a Cy5-labeled secondary antibody, washed, dried, scanned, and the fluorescence intensity of the spots was measured. The concentrations of MBP-30aa and PKCβ were 2.98 μM and 15.3 nM, respectively. The *error bars* represent the difference of tetraplicates in the experiment

ylated PKCβ bind to the chip and react to the antibody. We are making efforts to reduce this background.

Unlike the previous report which developed a fluorescence polarization assay of protein kinase C using Cy5-labeled phosphoserine antibody [20], in this study, phopho-Ser/Thr antibody and Cy5-labeled second antibody were employed.

To confirm the effectiveness of the PKCβ protein chip as an inhibitor screening tool, two well-known PKCβ inhibitors such as TMP and bisindolylmaleimide I were used. As shown in Fig. 7, both TMP and bisindolylmaleimide I inhibited PKCβ activity in the protein chip.

In conclusion, we have developed a fluorescence-based HTS methodology for the screening of depigmenting agents. This screening method allows for an easy, fast, and compact strategy. We have also demonstrated that this method is dependent on the concentration of PKCβ and MBP-30aa. This approach could be possibly used to screen inhibitors of PKCβ. These results suggest the potential of PKCβ protein chip as a HTS tool in the screening of depigmenting agents.

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