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Identification of a Rapidly Dephosphorylating 95-kDa Protein as Elongation Factor 2 during 8-Br-cAMP Treatment of N1E115 Neuroblastoma Cells

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Summary: Treatment of 8-Br-cAMP promotes neurite outgrowth and neuronal differentiation in N1E115 mouse neuroblastoma cells. Prior or simultaneous treatment of PMA blocks 8-Br-cAMP-mediated neurite outgrowth. Phosphorylation of cellular proteins during these treatments was examined in a permeabilized cell system. While PMA promotes phosphorylation of the heat-stable protein kinase C substrates MARCKS and neuromodulin, 8-Br-cAMP hastens the dephosphorylation of a protein of M_r 95k (p95). Extensively purified, N-terminal sequenced, and judged from its phosphorylation properties, p95 was identified as the eukaryotic elongation factor-2 (eEF-2), whose dephosphorylation has been reported to be related to an increase in protein synthesis. It is likely 8-Br-cAMP stimulates dephosphorylation of eEf-2, promotes protein synthesis that eventually leads to neuronal differentiation in N1E115 cells.

The N1E115 clonal cells, derived from a spontaneously arising murine neuroblastoma C1300 (1), have been widely used to study the differentiation of developing neurons. These cells differentiate morphologically (extending neurite outgrowth [NOG]) when grown in a serum deprived medium (2). The NOG of N1E115 as well as other neuroblastoma cells, such as human SH-SY5Y, are especially facilitated by the presence of a cell permeable cAMP analogue, such as 8-Br-cAMP, an activator of cAMP-dependent protein kinase (PKA) (3). Brief prior or simultaneous treatment of these cells with a tumor-promoting phorbol ester, such as PMA, an activator of protein kinase C (PKC), completely inhibited the cAMP-mediated NOG (4). Prolonged PMA treatment, which downregulates PKC, or PKC inhibitor, however, reduces such inhibition and stimulates NOG (5-7). The present communication describes our attempt to understand the biochemical mechanisms underlying the cAMP/PKA induced NOG and the

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antagonism by PMA/PKC by probing the phosphorylation reactions. In a permeabilized N1E115 cell system, treatment with 8-Br-cAMP resulted in a rapidly dephosphorylation of a protein with an apparent M_r of 95,000 (p95) on SDS-PAGE. The p95 protein was extensively purified and its N-terminal 15 amino acids was found to match those of human and rat elongation factor 2 (eukaryotic, eEF-2) (8). It has been reported that dephosphorylation of eEF-2 by protein phosphatase 2A (PP2A) stimulates translational elongation, while its phosphorylation by Ca^{2+}/CaM-dependent protein kinase III (PKIII) inhibits protein synthesis (9-12). It appears that the cAMP stimulated NOG and differentiation in N1E115 cells also promotes dephosphorylation of EF-2 leading to a stimulated protein synthesis machinery.

Experimental Procedures

N1E115 mouse neuroblastoma cells were maintained in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100μg/ml streptomycin at 37^{0}C in a humidified atmosphere of 90% air and 10% CO_2. Cells were seeded into 6-well plates and grown for at least 2 days before experiment. Prior to phosphorylation, cultures were kept in serum-free medium for 2 h. Cells were then washed twice with physiological saline consisting of (in mM) 125 NaCl, 5 KCl, 25 HEPES (pH 7.4), 1.2 $MgSO_4$, 1.2 Na_2HPO_4, 2.5 $CaCl_2$. Phosphorylation was commenced with 20μM [γ-^{32}P]ATP (specific activity ~3,000 cpm/pmol) and 40 μM digitonin in a 0.4 ml solution consisting of (in mM) 139 K-glutamate, 1 $MgSO_4$, 1 K_2HPO_4, 10 PIPES (pH 7.0), 1 EDTA, 1 $CaCl_2$ and the test reagent as indicated. Reactions were carried out for 10 min and were stopped by the addition of NP-40, EDTA, and NaF to a final concentration of 1%, 10 mM and 100 mM, respectively. Extracted proteins were analyzed by SDS-PAGE and autoradiography.

Proteins extracted from 12 150-mm dishes of cells, which were phosphorylated as described above but with, except two dishes, cold ATP, were separated on a DE52 (2x6.5 cm) ion exchange column equilibrated with buffer A (20 mM Tris-Cl, pH 7.5, containing 1 mM DTT, 2 mM EDTA and 5 mM EGTA). After washing, proteins were eluted with a 0-0.4 M KCl gradient, and the effluent fractions were analyzed by SDS-PAGE and autoradiography. Fractions containing p95 were combined, concentrated, and loaded onto a Bio-Gel A-0.5m (1x40 cm) gel filtration column in buffer A. Fractions were analyzed and those containing p95 were concentrated, separated on SDS-PAGE, transferred to Immobilon P membrane, and the protein band was excised for microsequencing.

Results and Discussion

N1E115 neuroblastoma cells sprout short neurites when cultured in serum free environment. Treatment of cells with 8-Br-cAMP (1 mM), NOG becomes more elaborated within just 30 min. If cells were exposed to PMA (10-1000nM) briefly (10 min) before or simultaneously with 8-Br-cAMP, then NOG was completely inhibited. These morphological changes will be described elsewhere in detail (H. Li and F. L. Huang, in preparation). Changes in PKA and PKC activities in the extracts of 8-Br-cAMP- and PMA- treated cells were shown in Fig. 1. Assay with peptide MBP_{4-14}, a specific substrate of PKC, treatment of PMA promotes a decrease of cytosolic PKC activity but an increase of the particulate activity (Fig. 1A). Such translocation of PKC activity persists even in the presence of 8-Br-cAMP. On the other hand,

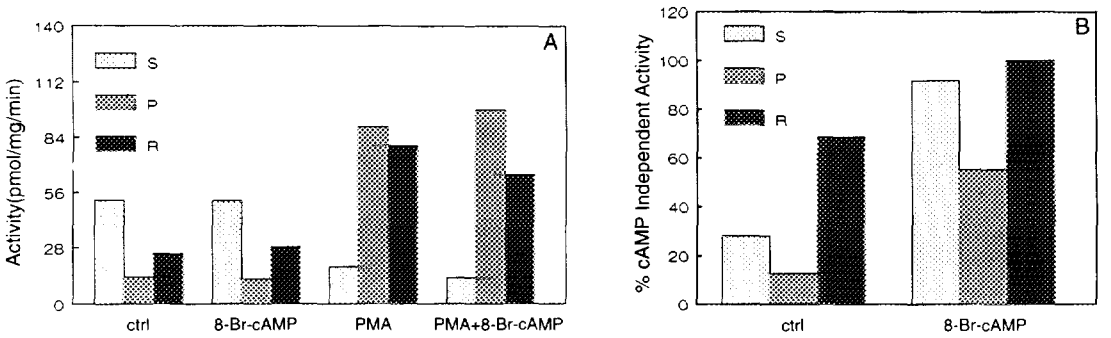

Fig. 1. Protein kinase C and protein kinase A activities in the extracts of N1E115 cells. Cells were kept in serum-free medium for 2 h before treating 8-Br-cAMP (1 mM), or PMA (1 μM), or both for 30 min. After removing the medium, cells were washed three times with cold Tris (20 mM)-buffered saline and kept frozen at -70^{0}C. Cells were then scraped into buffer of 20 mM Tris.Cl, pH 7.5, containing 1 mM DTT, 2 mM EDTA, 5 mM EGTA, 10 % glycerol and 50 μM AEBSF, and sonicated (2x15sec). The soluble (S) fraction was obtained by centrifugation at 100,000xg, the pellet was sonicated in the same buffer but containing 0.5% NP40 and centrifuges again to give detergent extract (P), and the residual pellet (R) was resuspended. PKC was assayed by the phosphorylation of MBP_{4-14} in a reaction mixture of 25 μl containing 30 mM Tris.Cl, pH 7.5, 6 mM MgOAc, 120 μM [γ-^{32}P]ATP(~ 1000cpm/pmol) , 100 μg/ml PS, 20 μg/ml DG, and 0.4 mM $CaCl_2$ or 1.5 mM EGTA to substitute for the activator. After 10 min incubation, protein was precipitated and the supernatant was spotted on P81 paper to determine the peptide bound radioactivity. PKA was assayed by the phosphorylation of Kemptide in the similar reaction mixture containing with or without 10 μM cAMP as activator.

treatment with 8-Br-cAMP evokes an increase in cAMP-independent PKA activity on Kemptide, a PKA-specific peptide substrate (Fig. 1B).

To determine whether activation of PKC or PKA affected the phosphorylation of cellular proteins, phosphorylation reactions were carried out in permeabilized cells with exogenously added [γ-^{32}P]ATP. Phosphorylated proteins were resolved on SDS-PAGE for autoradiography as shown in Fig. 2. Under PMA stimulation (left hand panel), there is a slight increase in the total ^{32}P incorporation but the major radioactive protein band is similar to that of the un-treated cells. Stimulation of PKA, unexpectedly did not show an increase in the total ^{32}P incorporation, whereas a major phosphoprotein, having an apparent M_r of 95,000 (p95), showed a decrease in phosphorylation. Okadaic acid alone, or okadaic acid and PMA increase the phosphorylation of p95. This protein was not heat-stable, a property that often observed for PKC substrates. As a matter of fact, right hand panel of Fig. 2 shows that heat treatment has eliminated p95 and the heat-stable protein profile showed increased phosphorylation of MARCKS and neuromodulin, specific PKC substrates, in the PMA, okadaic acid, and okadaic acid and PMA treated cells. The middle panel of the figure indicates that the dephosphorylation of p95 is 8-Br-cAMP dose dependent but is not influenced by the presence of PMA up to 1 μM.

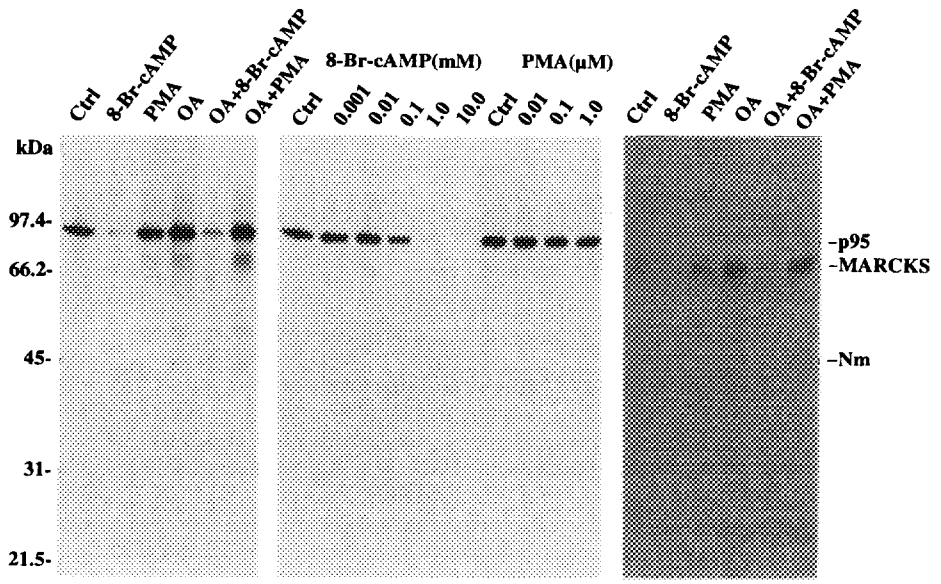

Fig. 2. Phosphorylation of cellular proteins in the permeabilized N1E115 cells. Cells were treated, phosphorylated, and phosphorylated proteins analyzed as described in the Procedure. Left panel shows the effect of the reagents when tested at 1 mM 8-Br-cAMP, 1 μM PMA or 25 nM okadaic acid or their combination. The middle panel shows the phosphorylation at various concentrations of 8-Br-cAMP or PMA. The right panel shows the heat stable proteins (supernatant of 10 min 90^{0}C treatment) from samples of the left-hand panel.

Phosphorylated proteins from non-treated cells were separated on DEAE-cellulose (DE52) column. Fig. 3 shows protein staining (A) and autoradiography (B) of the SDS-PAGE separated proteins of DE52 fractions. There is only one radioactive band that coincides with a protein band in fractions 11-23. Fractions 11-19 were pooled and concentrated by Centricon 10 before separating on a Bio-Gel A-0.5m gel filtration column. First half portion of the fractions were analyzed and shown in Fig. 3C (protein) and 3D (autoradiography). Fractions 18 and 19 that were eluted in the void volume contains p95 protein band which corresponds to the radioactive band. Though the fractions were heterogeneous, p95 was well-separated from other contaminants allowing excision from the Immobilon membrane for the N-terminal sequencing.

The 15 N-terminal amino acid sequence of p95, N-Val-Asn-Phe-Thr-Val-Asp-Gln-Ile-Arg-Ala-Ile-Met-Asp-Lys-Gly, matches exactly with that of elongation factor -2 from human and rat. Eukaryotic elongation factor-2 (eEF-2) is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase III (PKIII) (9,12), and the phosphorylated eEF-2 is dephosphorylated by protein phosphatase 2A (PP2A) (10,11). To confirm that p95 is eEF-2 we tested the influence of various agents on its phosphorylation in N1E115 cells. As shown in Fig. 4, in the presence of EGTA and TFP, the inhibitors of PKIII, phosphorylation of p95 was greatly inhibited, whereas in the presence of staurosporine, calphostin, and genistein, inhibitors of other protein kinases,

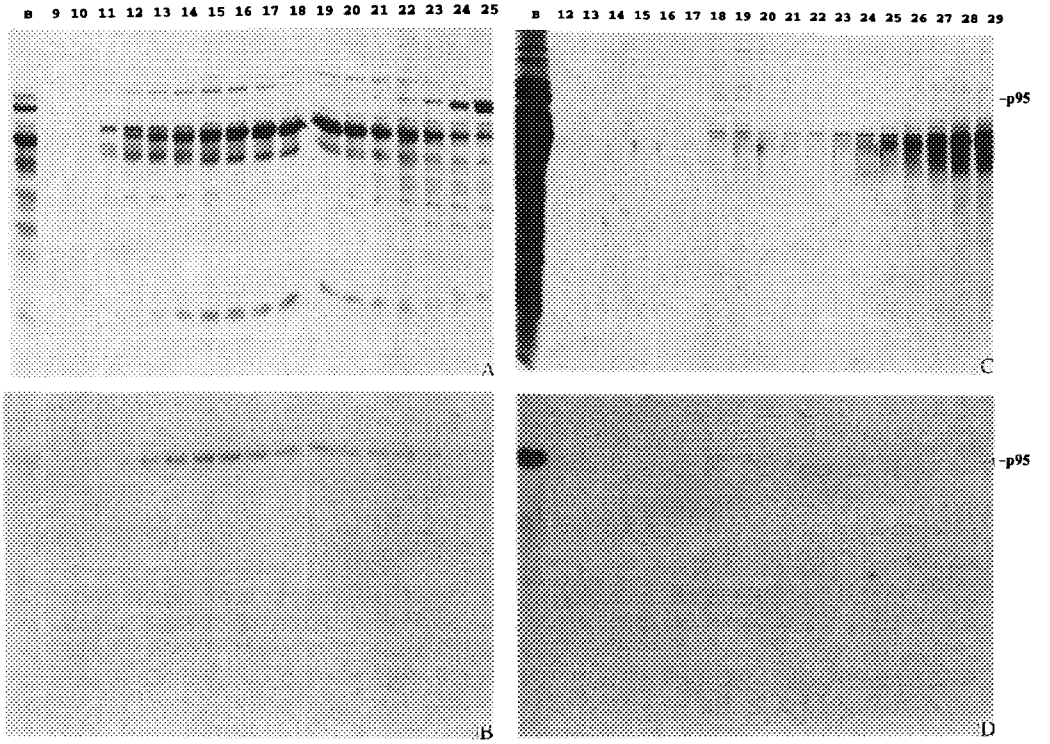

Fig. 3. DE52 ion exchange column chromatography (A and B) and A-0.5M gel filtration for the purification of p95. Chromatography and SDS-PAGE are as described in the Procedure. A and C are Coomassie brilliant blue R250-stained protein patterns, B and D are their corresponding autoradiographies. In both columns, only portions of the analyses were shown, fraction numbers were as indicated at the top. B denotes samples before fractionation.

inhibition was not apparent. In the presence of okadaic acid, an inhibitor of protein phosphatases, especially potent against PP2A, the phosphorylation of p95 was enhanced. However, it was again dephosphorylated by the addition of 8-Br-cAMP. Intriguingly, dephosphorylation induced by cAMP was not affected by cycloheximide, the protein synthesis inhibitor, while cycloheximide alone did not produce such an effect.

These results point to the identity of p95 as the eEF-2. Dephosphorylation of p95 induced by the treatment of cAMP promotes protein synthesis, however, dephosphorylation of p95 as well as NOG occur in the presence of protein synthesis inhibitor, cycloheximide (Li and Huang, unpublished). It is likely that NOG, which is evident within 30 min of treatment, is independent of protein synthesis, while the long term effect of cAMP-induced neuronal differentiation is dependent on protein synthesis. Other studies have also indicated that initial morphological differentiation (NOG) is a manifestation of microtubule rearrangement without requiring newly synthesized protein (13,14).

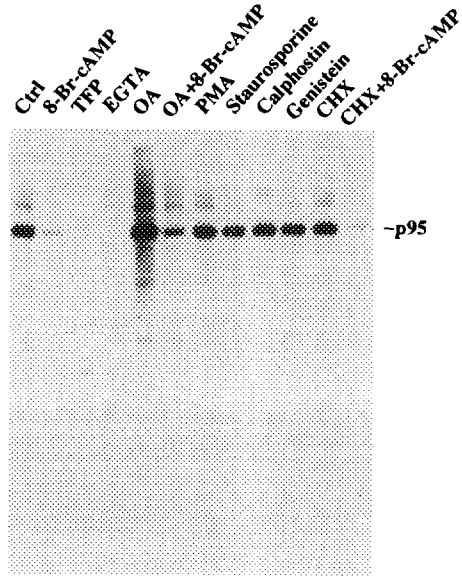

Fig. 4. Effects of protein kinase inhibitors on the phosphorylation of p95 in the permeabilized N1E115 cells. Phosphorylation reactions were as described in the Procedure. Testing reagents were used at the following concentrations: 8-Br-cAMP 1mM, TFP 160μM, EGTA 10 mM, okadaic acid 20 nM, PMA 1μM, staurosporine 214 nM, calphostin 800 nM, genistein 20 μM, and cycloheximide (CHX) 10 μg/ml.

The mechanism behind the dephosphorylation of p95 as induced by the 8-Br-cAMP treatment is not readily clear. Since dephosphorylation of p95 *in vitro* is most actively carried out by PP2A, it is reasonable to speculate that in these cells activation of PKA may in some way indirectly activate PP2A. In Fig. 4, the presence of okadaic acid alone, which inhibited protein phosphatases (specifically PP2A), phosphorylation of p95 was actually more than that of non-treated cells. Addition of 8-Br-cAMP in the presence of okadaic acid not only did not retain the phosphorylation, but on the contrary promoted the dephosphorylation, possibly due to the activation of protein phosphatase that overcame the inhibition by okadaic acid. It has been reported that activation of PKA leads to the inhibition of protein phosphatase 1 through the phosphorylation and activation of phosphatase inhibitor-1 (15). A mechanism for inhibition of PP2A by cAMP/PKA has also been suggested in the adrenaline-induced inactivation of acetyl-CoA carboxylase (16). *In vitro*, cAMP/PKA can phosphorylates and activates PKIII leading to phosphorylation and inactivation of eEF-2 (17). In a cell-free translation system of reticulocyte lysate, cAMP activates protein synthesis through dephosphorylation of eEF-2 (18). In these N1E115 neuroblastoma cells, whether cAMP, by activating PKA, can stimulate PP2A and results in the subsequent dephosphorylation of eEF-2 warrants further investigation.

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