Elongation factor-2 kinase: immunological evidence for the existence of tissue-specific isoforms

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Abstract eEF-2 kinase is a ubiquitous Ca^{2+}/calmodulin-dependent protein kinase that is specific for protein synthesis elongation factor-2 (eEF-2). This study describes an improved procedure for the purification of eEF-2 kinase from rabbit reticulocyte lysate. The eEF-2 kinase preparation was used to raise polyclonal antibodies, which immunoprecipitated eEF-2 kinase protein and activity from rabbit reticulocyte lysate. The antibodies recognized a single 103 kDa band in extracts from several cell lines including NIH 3T3, PC12, C6 glioma, HeLa, and MCF-7 breast carcinoma. However, there was no immunoreactivity in extracts of rabbit or bovine liver or rabbit kidney despite the presence of abundant eEF-2 kinase activity in these tissues. Exposure of PC12 cells to nerve growth factor (NGF) resulted in rapid down-regulation of eEF-2 kinase activity and a decrease in immunoreactivity. After 24 h of incubation with NGF, the activity of the kinase recovered to 80% of initial values. In contrast, the immunoreactivity of eEF-2 kinase continued to decrease. These data suggest that tissue-specific isoforms of eEF-2 kinase may exist and that these isoforms may be regulated by growth factors.

Key words: Elongation factor-2; Protein synthesis; Protein kinase

1. Introduction

Elongation factor 2 kinase (eEF-2 kinase), also known as Ca^{2+}/calmodulin-dependent protein kinase III, has been shown to phosphorylate eukaryotic elongation factor-2 (eEF-2) with exceptional specificity reviewed in [1,2]. eEF-2 is a 100 kDa polypeptide that catalyzes ribosomal translocation, a reaction that results in movement of the ribosome along mRNA during translation. Upon phosphorylation, eEF-2 becomes inactive, resulting in the arrest of protein synthesis [3,4].

Phosphorylation of eEF-2 occurs during cellular stimulation by mitogens, activation of neurons, and stimulation of secretory cells by various secretogogues [1,2]. The activation of eEF-2 kinase and transient arrest of protein synthesis may therefore act to conserve cellular energy and/or remove short-lived repressors as a part of a larger signal transduction pathway. In addition, the activity of the kinase is markedly increased in malignant cell lines [5,6].

Despite the importantance of eEF-2 kinase, relatively little is known about its activation and regulation. Progress has been hampered, at least in part, by the inability to purify sufficient quantities of the enzyme to raise antibodies and undertake detailed analysis.

Here we describe a rapid and efficient procedure for purification of eEF-2 kinase. Using the purified material, we obtained polyclonal antibodies to the rabbit reticulocyte eEF-2 kinase. We used these antibodies to identify the tissue distribution and regulation of eEF-2 kinase in PC12 cells, and compared these results to those obtained by measuring enzyme activity. We present evidence that in several tissues of the rabbit and during neuronal differentiation of PC12 cells, there exists eEF-2 kinase activity not recognized by antibodies raised against rabbit reticulocyte eEF-2 kinase.

2. Materials and methods

Rabbit reticulocyte lysate was obtained from Green Hectares, Oregon, WI. Bovine liver was obtained from Cartaret Arbitrage, Cartaret, NJ. Fetal bovine serum (FBS), horse serum, DMEM, and nerve growth factor (NGF) were obtained from Gibco. DEAE-Sephacel and all buffers and chemicals were obtained from Sigma. Phenyl Sepharose and Mono-Q columns were obtained from Pharmacia.

PC12 cells (kindly provided by Erick Morris, Robert Wood Johnson Medical School) were cultured in DMEM containing 10% horse serum and 5% FBS in flasks precoated with 500 μg/ml collagen in 10% acetic acid. NIH 3T3, C_6 glioma cells, and Rat Embryo Fibroblasts (REF) were cultured in DMEM containing 10% FBS. CV-1 cells were cultured in EMEM with Earle's balanced salt solution containing 10% FBS. HeLa and MCF-7 cells were cultured in Alpha-MEM with non-essential amino acids containing 10% FBS. T98G cells were cultured in Ham's F-10/DMEM (1 : 10) plus 10% FBS. All cultures were maintained in a humidified incubator at 37°C, in an atmosphere of 95% air, 5% CO_2. Cells were checked regularly and found to be free of contamination by mycoplasma or fungi.

Tissues were excised from rabbits and immediately homogenized on ice in Buffer D using a Dounce homogenizer; Buffer D contained 25 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM sodium pyrophosphate, 2 mM EDTA, 3 mM EGTA, 0.5 mM PMSF, 1.25 μg/ml leupeptin, 1.25 μg/ml pepstatin-A, and 2.5 μg/ml soybean trypsin inhibitor. Homogenates were centrifuged in an Eppendorf centrifuge for 30 min at 4°C and the supernatant decanted. This material was stored at −80°C in 100 μl aliquots. eEF-2 kinase was partially purified by adding 1 vol. of a DEAE-Sephacel slurry equilibrated in 20 mM Trizma base, pH 7.4, 1 mM $MgCl_2$, 10% glycerol, 7 mM β-mercaptoethanol, 2 μg/ml leupeptin, and 2 μg/ml pepstatin-A. The slurry was incubated at 4°C on a rotary shaker for 30 min and the Sephacel pelleted by gentle centrifugation. After several washes with Buffer D, the supernatant was decanted and an initial fraction (not containing eEF-2 kinase) was eluted with two washes of 3 vol. of Buffer D containing 250 mM NaCl. The fraction containing eEF-2 kinase was eluted with 1 vol. (typically 100 μl) of Buffer D containing 500 mM NaCl. The yields were sufficient from this procedure to measure enzyme activity and carry out immunoassays as described below.

eEF-2 kinase was purified from rabbit reticulocyte lysate and bovine liver. The procedure was the same for both sources with modifications as noted. Rabbit reticulocyte lysate (250 ml) was centrifuged at 50000×*g* for 30 min and added to 100 ml of a cold slurry of

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DEAE-Sephacel equilibrated in 20 mM Trizma base, pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 7 mM βME, 2 μg/ml leupeptin, and 5 μg/ml pepstatin (Buffer A). The slurry/lysate was continuously stirred at low speed for 30 min at 4°C. After extensive washes with Buffer A to remove unbound material, the slurry was poured into a 100 ml XK-26 column (Pharmacia) and equilibrated to baseline at 4°C on a Pharmacia FPLC system with Buffer A. A 50–600 mM NaCl linear gradient in Buffer A was applied and eluted fractions were tested for eEF-2 kinase activity. For purification of the kinase from bovine liver, we used a 50–1000 mM NaCl gradient. Fractions containing kinase activity were pooled, dialyzed overnight on ice against 20 mM Trizma base, pH 7.0, 1.0 M $(NH_4)_2SO_4$, 10% glycerol, 7 mM βME, 2 μg/ml leupeptin, and 5 μg/ml pepstatin (Buffer B), and applied to a 1.0 ml HS Phenyl Sepharose column (Pharmacia). The column was developed with a dual 1.0–500 mM and 500 mM–0.0 M $(NH_4)_2SO_4$ linear gradient. Fractions containing kinase activity were pooled and applied directly to a HR5/5 Mono-Q column (Pharmacia) equilibrated with 20 mM Trizma base, pH 7.4, 1 mM $MgCl_2$, 10% glycerol, 7 mM βME, 2 μg/ml leupeptin, and 5 μg/ml pepstatin A. The column was developed with a dual 0.0–300 mM and 300–700 mM KCl linear gradient. eEF-2 kinase was recovered as a homogeneous species in a total volume of 1.0 ml (Table 1).

Phosphorylation of eEF-2 in eluted fractions and lysates was measured in 40 μl reaction volumes containing 50 mM HEPES, pH 7.4, 10 mM magnesium acetate, 0.1 mM $CaCl_2$, 5 mM dithiothreitol, 15 μM ATP, 2 μCi [γ-^{32}P]ATP, 1 μg calmodulin, 0.5 μg rabbit reticulocyte eEF-2, and 0.5–100 μg of sample. Reactions were initiated by adding the reaction mixture to the FPLC fraction or tissue sample and incubating at 30°C for 2 min. Assays were terminated by adding 20 μl of 3 times Laemmli sample buffer containing 150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 15% β-mercaptoethanol, and 0.003% bromphenol blue. Samples were boiled for 5 min and proteins separated by 8% SDS-PAGE. Phosphoproteins were analyzed by autoradiography. For quantitative analysis of eEF-2 phosphorylation, gels were scanned by an Ambis Radioanalytic Imaging System (Ambis, Inc., San Diego, CA) and specific activity calculated from background-corrected data.

To generate antibodies, purified eEF-2 kinase was electrophoresed on an 8% SDS-PAGE gel and briefly stained with Coomassie blue R-250. The kinase band was excised (approx. 0.5 μg total protein) and suspended in 300 μl of PBS. This material was passed through successively smaller bore needles until it passed easily through a 26-ga needle. Two 4–6-week-old Balb/C mice were immunized intraperitoneally with 50 μl of gel suspension and then boosted twice at 7 day intervals. At days 8–10 after the last boost, mice were bled via a tail vein and sera stored at −40°C. Antibody titer was determined by immunoblot analysis. Control sera was obtained from the same mice 3 days prior to the primary immunization.

Immunoblotting with anti-eEF-2 kinase was performed according to the method of Towbin et al. with minor modifications [7]. Proteins were separated by 8% SDS-PAGE and then transferred using a semi-dry electroblotting apparatus (Owl Scientific) to nitrocellulose at 13.8 V for 2 h. The membranes were blocked in 10 ml of PBS containing 5% non-fat dry milk (NFDM) for at least 1 h. The nitrocellulose was incubated with anti-eEF-2 kinase antisera in PBS with 1% NFDM at 4°C overnight. Immunoreactive protein was detected by goat anti-mouse antibodies conjugated to horseradish peroxidase. An ECL chemiluminescence detection system (Amersham) was used for antigen detection as per the manufacturer's protocol. Immunoprecipitations with control (pre-immune) and anti-eEF-2 kinase antisera were done by incubating 100 μl of sample with 30 μl of sera for 1 h at 4°C on a vortex shaker. Immune complexes were recovered by adding 50 μl of a protein G-Sepharose (Sigma) slurry and incubating as described above. The beads were recovered by gentle centrifugation and washed 3 times with PBS, boiled in Laemmli solubilizing buffer and centrifuged briefly. The resulting supernatants were run on 8% SDS-PAGE gels and immunoblotted as described above. Immunoprecipitation and recovery of eEF-2 kinase activity was done as follows: samples were immunoprecipitated as described above except after incubation with beads, a kinase assay was performed on both the supernatant and the beads. In the latter case, the whole volume of Sepharose was incubated under standard assay conditions except that time was extended to 5 min.

PC12 cells were grown in T-75 culture flasks and treated with 50 ng/ml NGF for various periods of time. At the experimental times indicated, media was aspirated and cells detached by trituration with cold, sterile PBS containing 5 mM EDTA and washed twice by centrifugation in ice-cold PBS. Cell pellets were homogenized in a Dounce homogenizer in Buffer D. Homogenates were centrifuged in an Eppendorf centrifuge for 30 min and the protein concentration of the supernatant was determined by BioRad protein assay. This material was used in both immunoblotting and eEF-2 kinase activity assays.

Table 1
eEF-2 kinase purification from rabbit reticulocyte lysate

Purification step	Protein (mg)	Specific activity (pmol/min/mg)	Recovery (%)
50 000 × g	12915	44.9	100
DEAE Sephacel	78.2	3174	38
Phenyl Sepharose	3.5	37 569	20
Mono-Q	0.033	3×10^6	16

Protein concentration was measured by BioRad protein assay. Specific activity was determined by calculating ATP incorporated per minute per mg protein into eEF-2 using an Ambis radioanalytic imaging system. See Section 2 for details of the purification scheme.

3. Results

To determine the tissue distribution of eEF-2 kinase, various rabbit tissues were screened for eEF-2 kinase activity. Tissue extracts were obtained as described in Section 2. Ex-

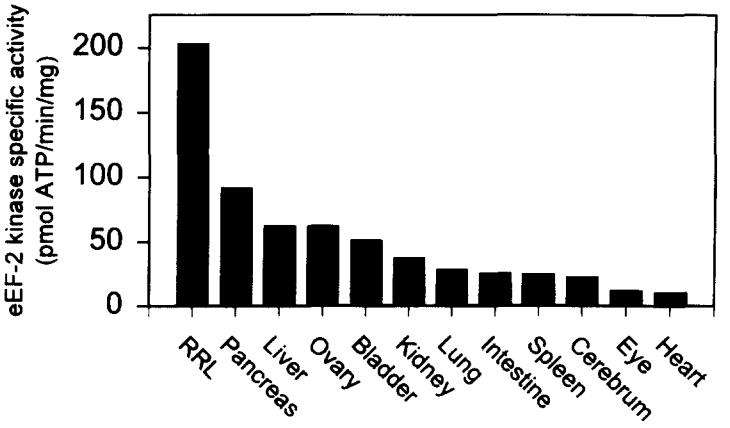

Fig. 1. eEF-2 kinase activity in various rabbit tissues. Tissues were semi-purified with DEAE-Sephacel and protein concentrations adjusted to 20 μg/5 μl. Activity was assayed as described in Section 2 and eEF-2 phosphorylation quantitated on an Ambis radioanalytic imaging system.

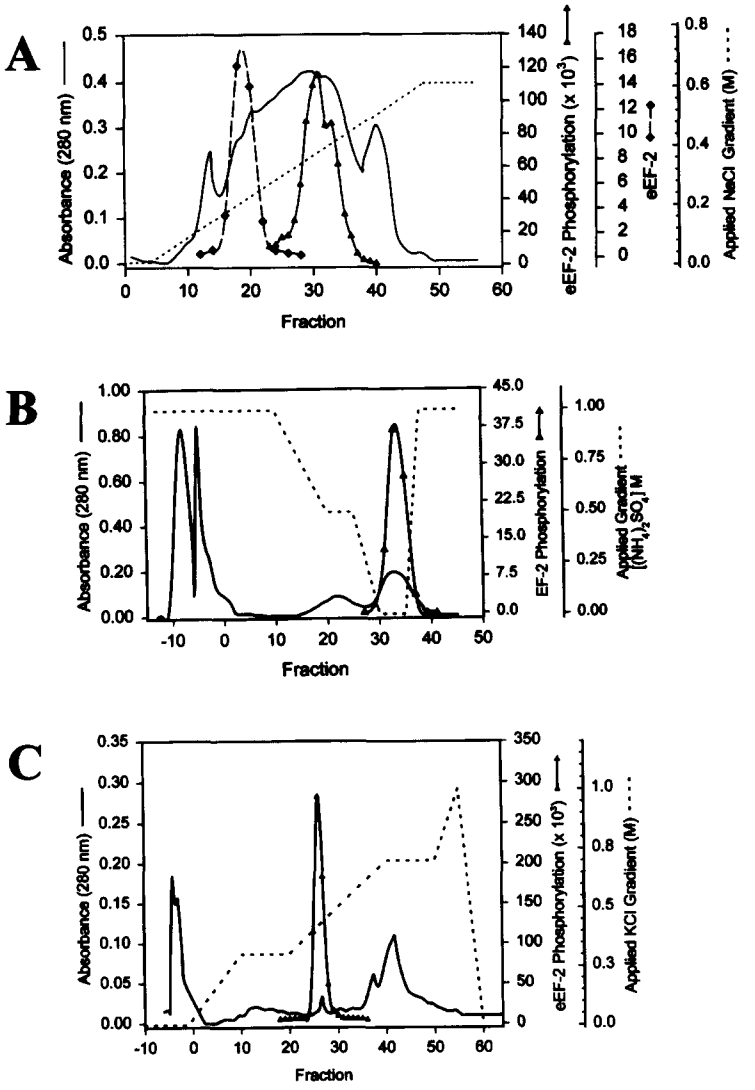

Fig. 2. Purification of eEF-2 kinase from rabbit reticulocytes. A: DEAE-Sephacel chromatography of 250 ml (13 g protein) rabbit reticulocyte lysate. A linear gradient of 50–600 mM NaCl was applied in 5 column volumes. (♦, eEF-2; ▲, eEF-2 kinase). The eEF-2 was further purified and used as a substrate. B: Phenyl Sepharose chromatography of DEAE-eluted material. Peak fractions were dialyzed against Buffer B prior to loading. C: Mono-Q chromatography of eEF-2 kinase from phenyl Sepharose fractions. Phenyl Sepharose-eluted material was applied directly to the column and eluted with a dual step-linear gradient as shown.

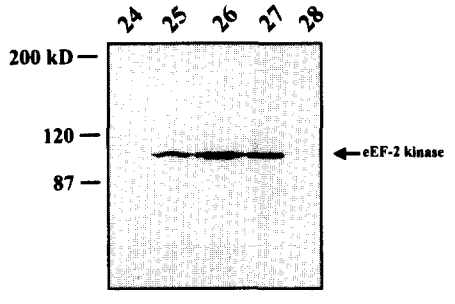

Fig. 3. SDS-PAGE of Mono-Q fractions from eEF-2 kinase purification. Fractions 24–28 were concentrated 2-fold in a Centriprep Concentrator (Amicon) and run on a 8% gel stained with Coomassie Blue R-250. The arrow indicates the 103 kDa eEF-2 kinase. eEF-2 phosphorylation assays of these fractions resulted in a corresponding peak of activity at fraction 26.

tracts from each tissue were chromatographed on DEAE cellulose to partially purify the kinase. Fig. 1 demonstrates that the highest activity of eEF-2 kinase was in rabbit reticulocytes, followed by pancreas, liver, ovary, bladder, and kidney.

Since rabbit reticulocyte lysate had the greatest specific activity of the kinase, we used this material for purification. After a 50000×*g* centrifugation, lysate was applied to DEAE-Sephacel and eluted with a linear gradient of NaCl (0.05–0.6 M) (Fig. 2A). The kinase was eluted as a single peak at approximately 0.4 M NaCl and was well separated from eEF-2. Fractions containing eEF-2 kinase were pooled, dialyzed against Buffer B, and applied to a HS Phenyl Sepharose column. The kinase activity was eluted with a two-step linear $(NH_4)_2SO_4$ reverse gradient (Fig. 2B) and fractions containing kinase activity applied directly to a Mono-Q anion exchange column. We found that the two-step linear gradient (Fig. 2C) gave the greatest separation of the kinase from other proteins.

The specific activity and yield of eEF-2 kinase at each step

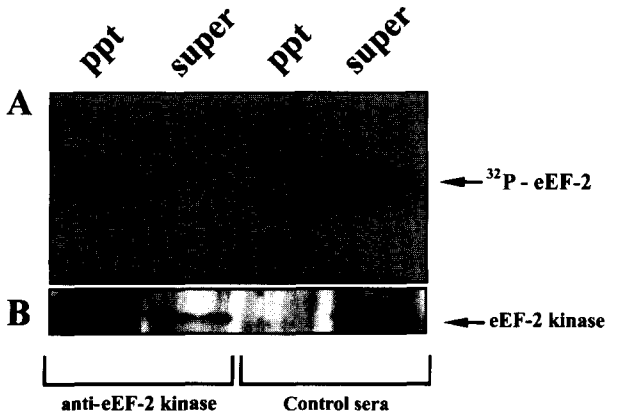

Fig. 4. Immunoprecipitation of rabbit reticulocyte lysate eEF-2 kinase. Enzyme recovered from the second stage of the purification (phenyl Sepharose) was precipitated with eEF-2 kinase antisera. A: Recovery of eEF-2 kinase activity from the immunoprecipitates. Polyclonal antisera was incubated with semi-purified eEF-2 kinase. Immune complexes were precipitated with protein G-Sepharose. Precipitated beads (ppt) and supernatant (sup) were incubated under standard kinase assay conditions (see Section 2) for 5 min. The panel is an autoradiograph of the reaction mixtures electrophoresed on an 8% SDS-PAGE gel. The arrow indicates phosphorylated eEF-2. Note that nearly all activity is recovered in the anti-eEF-2 kinase precipitate while the control sera contains activity only in the non-precipitated fraction. B: An immunoblot of SDS-PAGE-separated immunoprecipitates. Semi-purified eEF-2 kinase was incubated as above with both anti-eEF-2 kinase antisera and control antisera, and electrophoresed on an 8% SDS-PAGE gel. Proteins were transferred to nitrocellulose and the blot incubated with anti-eEF-2 kinase antiserum. The panel is an autoradiograph of an ECL immunoblot.

of purification are shown in Table 1. Using this procedure we were able to obtain approximately 30 μg of homogeneous eEF-2 kinase from 250 ml of lysate, with a yield of 16%. Purified eEF-2 kinase migrated as a single 103 kDa band by SDS-PAGE (Fig. 3).

Antibodies were generated as described in Section 2 and were able to detect as little as 1.6 ng of kinase by immunoblotting (data not shown). To confirm that the antibodies were specific for eEF-2 kinase, we performed immunoprecipitation experiments as shown in Fig. 4. Fig. 4A demonstrates the ability of the antibodies to immunoprecipitate enzyme activity, and Fig. 4B demonstrates the effects on the 103 kDa polypeptide. These data demonstrate that the polyclonal antibodies immunoprecipitated eEF-2 kinase and the immunoprecipitated material was able to phosphorylate eEF-2. Immunoprecipitation resulted in the disappearance of activity from the supernatant fraction. Control serum did not immunoprecipitate kinase activity nor did it precipitate the 103 kDa polypeptide.

We next compared the immunoreactivity of the kinase from several tissues and cell lines. The polyclonal antibodies readily detected the 103 kDa band in reticulocyte lysate but failed to detect this band in rabbit liver (Fig. 5A), even when equal amounts of eEF-2 kinase activity were loaded. In addition, these antibodies failed to recognize eEF-2 kinase in a preparation of purified kinase from bovine liver (Fig. 5A).

We next studied the immunoreactivity of the kinase in eight human, rat, monkey and mouse cell lines. Anti-eEF-2 kinase antibodies detected the 103 kDa protein in extracts of several different cell lines (Fig. 5B), including NIH 3T3 (mouse fibroblasts), HeLa (cervical carcinoma), MCF-7 (breast carcinoma), T98G (human glioblastoma), C6 (rat glioblastoma), and PC12 cells (rat pheochromocytoma). In contrast, the antibodies failed to recognize the 103 kDa band in the transformed monkey kidney fibroblast cell line CV-1, despite the presence of abundant kinase activity in extract from this cell line.

Treatment of PC12 cells with NGF produces a transient down-regulation of eEF-2 kinase activity [12–16]. We therefore studied whether or not the immunoreactivity of eEF-2 kinase in PC12 cells was also affected by NGF. The activity of the kinase declined to 5% of the initial value after 6 h of incubation with NGF and recovered to 80% of baseline values after 24 h. Immunoreactivity was also markedly decreased by 6 h. In contrast, at 24 h, immunoreactivity continued to decrease (Fig. 6B) despite the reappearance of kinase activity (Fig. 6A).

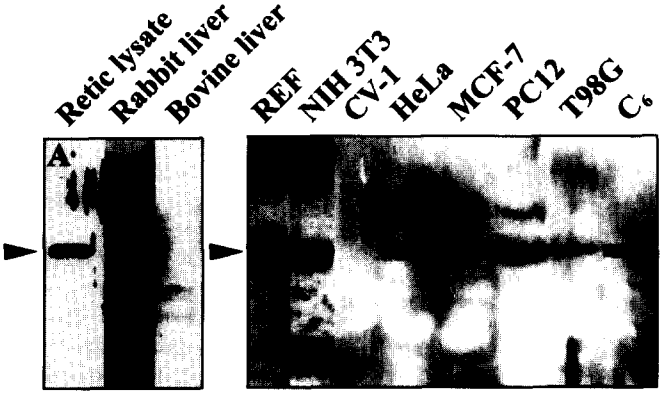

Fig. 5. eEF-2 kinase activity and protein expression. A: Immunoreactivity of eEF-2 kinase in rabbit retivculcuyte lysate, rabbit and bovine liver. Concentration of protein in reticulocyte lysate and rabbit liver extract was adjusted to load equivalent total enzyme activity in each lane. This required loading 3 times more protein from the rabbit liver extract compared to that of the reticulocyte lysate. The bovine liver material was extensively purified and at least 1 μg of eEF-2 kinase was used in the experiment. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with anti-eEF-2 kinase antiserum as described in Section 2. Immunoreactivity was assayed by ECL chemiluminescence. The arrow indicates eEF-2 kinase. B: Immunoreactivity of eEF-2 kinase from various cell lines. Tissue extracts (100 μg) prepared as described in Section 2 were assayed as in (A). REF, rat embryo fibroblasts; NIH 3T3, mouse fibroblasts; CV-1, transformed monkey kidney fibroblasts; HeLa, human cervical epithelioid carcinoma; MCF-7, human breast carcinoma; PC12, rat pheochromocytoma; T989G, human glioblastoma, C6, rat glioblastoma.

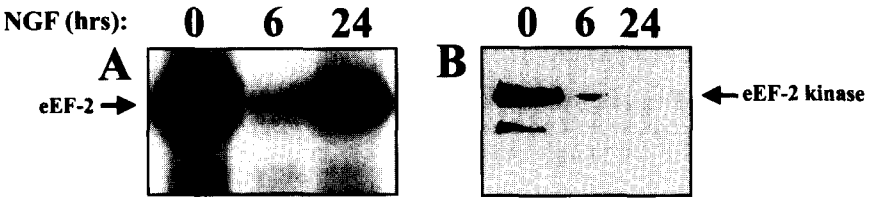

Fig. 6. Activity and immunoreactivity of eEF-2 kinase during PC12 cell differentiation. A: Equivalent protein concentrations were used in assaying eEF-2 phosphorylation for untreated (0), 6, and 24 h NGF treatment. The panel is an autoradiograph of kinase assay mixtures electrophoresed on 8% SDS-PAGE. B: Immunoreactivity of eEF-2 kinase in PC12 cell lysates treated with NGF. Equivalent protein amounts of cell lysates from (A) were run on an 8% SDS gel, transferred to nitrocellulose and incubated with anti-eEF-2 kinase antiserum.

4. Discussion

In this study we describe an improved procedure for the purification of eEF-2 kinase from rabbit reticulocyte lysate. The purification of this kinase from reticulocyte lysate has been previously described and had been suggested to be a 95–105 kDa polypeptide based on co-elution of kinase activity with a protein of within this molecular weight range [8,9]. Using newly generated polyclonal antibodies, we have shown that the 103 kDa band seen on SDS gels is eEF-2 kinase.

The purification procedure results in the recovery of approximately 30 μg of enzyme from 13 g of tissue, corresponding to 16% of the kinase activity in the starting material (Table 1). This represents a 10-fold increase in recovery compared to that of previous methods [8,9]. In this procedure, DEAE chromatography is used initially to facilitate removal of hemoglobin, which can account for up to 80% of the total protein in this tissue, and to separate the kinase from its substrate, eEF-2 (Fig. 2). The next step was purification on phenyl Sepharose. As shown in Fig. 2B, the majority of total protein is eluted in the unbound fraction while eEF-2 kinase remains tightly bound to the matrix. This level of purification was aided by the Mono-Q step and the selection of the gradient depicted in Fig. 2C, which separates the kinase from several co-migratory proteins such as hsp-90. The procedure resulted in the recovery of a single 103 kDa protein in three fractions as shown in Fig. 3.

Antibodies raised against purified eEF-2 kinase precipitate both eEF-2 kinase activity (Fig. 4A) and eEF-2 kinase protein (Fig. 4B). The antibodies also recognize eEF-2 kinase in cell lines from several species including mouse, rat, and human (Fig. 5).

In contrast, the antibodies do not recognize eEF-2 kinase in rabbit or bovine liver (Fig. 5A), despite the presence of abundant kinase activity. Although we considered several possible explanations for these results including interference of binding of antibody in different tissues, or differences in levels of expression of the kinase, these appeared unlikely since the experiments were done using partially purified materials under reducing conditions on SDS gels and in the presence of up to 3 μg of eEF-2 kinase (Fig. 5). These data raise the possibility of a different isoform of the kinase in liver that might account for the lack of antibody recognition in this tissue. A previous indication of the existence of eEF-2 kinase isoforms was provided by Knight et al. [10], who showed that Mn^{2+} stimulated phosphorylation of eEF-2 in the extracts of rat pancreatic acinar cells as well as in extract of whole pancreas, but not in the extracts from rat liver or kidney.

Further data supporting the existence of isoforms of eEF-2 kinase were obtained in PC12 cells. Treatment of PC12 cells with NGF induces differentiation into a neuronal phenotype, characterized by neurite outgrowth and the appearance of a number of molecular markers observed in sympathetic neurons [11]. More than a decade ago, Guroff and his colleagues studied the effect of NGF on the phosphorylation profile of extracts of PC12 cells [12,13]. They found that the only obvious difference in extracts prepared several hours after NGF treatment in comparison with untreated cells was the reduction in phosphorylation of eEF-2. It was found that the level of eEF-2 remains constant during incubation with NGF and the decrease in eEF-2 phosphorylation was due to down-regulation of the kinase activity [14]. We confirmed that treatment of PC12 cells with NGF leads to the down-regulation of eEF-2 kinase activity (Fig. 6A), which was due to the loss of the kinase rather than the substrate, eEF-2. The activity of eEF-2 kinase recovers to about 80% of the initial value by 24 h after incubation with NGF despite a persistant decrease in immunoreactivity (Fig. 6B). This is in agreement with previous observations by Nairn et al. [16] that recovery of kinase activity after NGF treatment requires new protein synthesis. In addition, these data suggest that the eEF-2 kinase, which reappears after 24 h of incubation with NGF, may represent an immunological variant different from that found in the undifferentiated cells. More detailed immunological and molecular studies should reveal whether the existence of different isoforms of eEF-2 kinase is due to post-translational modifications, alternative splicing, or the expression of different genes.

We have recently completed the cloning and sequencing of cDNAs for eEF-2 kinase from *C. elegans*, mouse and rat (Ryazanov et al. manuscript in preparation). Sequence analysis failed to reveal homology to the catalytic domains of other members of the protein kinase superfamily, with the exception of myosin heavy chain kinase-A from *Dictyostelium discoideum* [17]. Redpath et al. [18] have recently reported the sequencing of eEF-2 kinase from rat skeletal muscle, which is identical to what we have obtained from rat PC12 cells.

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