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A single step purification for recombinant proteins

Characterization of a microtubule associated protein (MAP 2) fragment which associates with the type II cAMP-dependent protein kinase

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A 167 base pair DNA cassette has been constructed to facilitate the detection and purification of recombinant proteins. This cassette, kfc, encodes three distinct peptide units: a phosphorylation site for the cAMP-dependent protein kinase (PKA), called kemptide, a factor Xa cleavage site, and a calmodulin-binding peptide. Expressed kfc fusion proteins can be purified from bacterial lysates in one step by affinity chromatography on calmodulin–agarose using EGTA as eluant. As a test of this system, we describe the expression, purification and characterization of the PKA binding domain of the microtubule associated protein (MAP 2).

Recombinant fusion protein; Affinity purification; Microtubule associated protein (MAP 2); cAMP-dependent protein kinase (PKA)

1. INTRODUCTION

One significant goal of applied gene technology is the production of proteins from recombinant DNA molecules. In recent years numerous plasmids, which efficiently express large quantities of recombinant proteins, have been marketed [1]. Detailed biochemical analysis of these recombinant proteins requires their purification from bacterial lysates, which can be a time-consuming and labor-intensive process. Several bacterial expression vectors, which simplify the purification of recombinant proteins from *E. coli*, have been constructed. For example, proteins of interest can be fused with β-galactosidase and then purified by substrate-affinity or immuno-affinity chromatography [2,3]. Other vectors direct the synthesis of polypeptides as fusion proteins with *Staphylococcus* protein A, which can be purified on IgG–Sepharose [4]. Disadvantages of these methods are (i) only limited amounts of pure material can be obtained and (ii) the denaturing conditions used during affinity elution steps can irreversibly alter biological activity of the protein products. To counteract these potential hazards, bacterial expression plasmids have been developed which allow the affinity purification of fusion proteins using non-denaturing conditions. For example, the pGEX system produces recombinant proteins fused to the C-terminus of the enzyme glutathione-*S*-transferase (GST) which can be purified by glutathione-affinity chromatography [5]. Alternative purification strategies involve the expression of proteins containing poly-arginine at their C-termini that can be purified by cation-exchange chromatography [6], or the production of proteins that are secreted into the periplasmic space or culture medium [7,8].

In this report we describe the construction of a DNA cassette, kfc, which encodes a 51 residue polypeptide comprised of three functional units, a phosphorylation site for the cAMP-dependent protein kinase (PKA) [9], a recognition site for the coagulation factor Xa [10,11] and a peptide derived from the C-terminus of skeletal muscle myosin light-chain kinase that binds calmodulin with nanomolar affinity [12,13]. When the kfc cassette is attached to the coding region of a cDNA, a calmodulin binding fusion protein is expressed which can be purified in one step by affinity chromatography on calmodulin–agarose. The fusion protein binds to the column in a calcium-dependent manner and is eluted with EGTA. After purification, the affinity tail can be removed by proteolysis with factor Xa. Detection of kfc fusion proteins is enhanced since the molecule can be specifically phosphorylated by PKA. To evaluate this sytem, we have expressed, purified and characterized MAP 2 (Δ1-154)-kfc, a protein fragment of the microtubule associated protein (MAP 2) which binds the type II regulatory subunit of PKA.

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2. MATERIALS AND METHODS

2.1. *Construction of the kfc cassette*

Construction of the kfc cassette was performed in three stages (Fig. 1A). First, the PKA phosphorylation unit, the kemptide sequence, was

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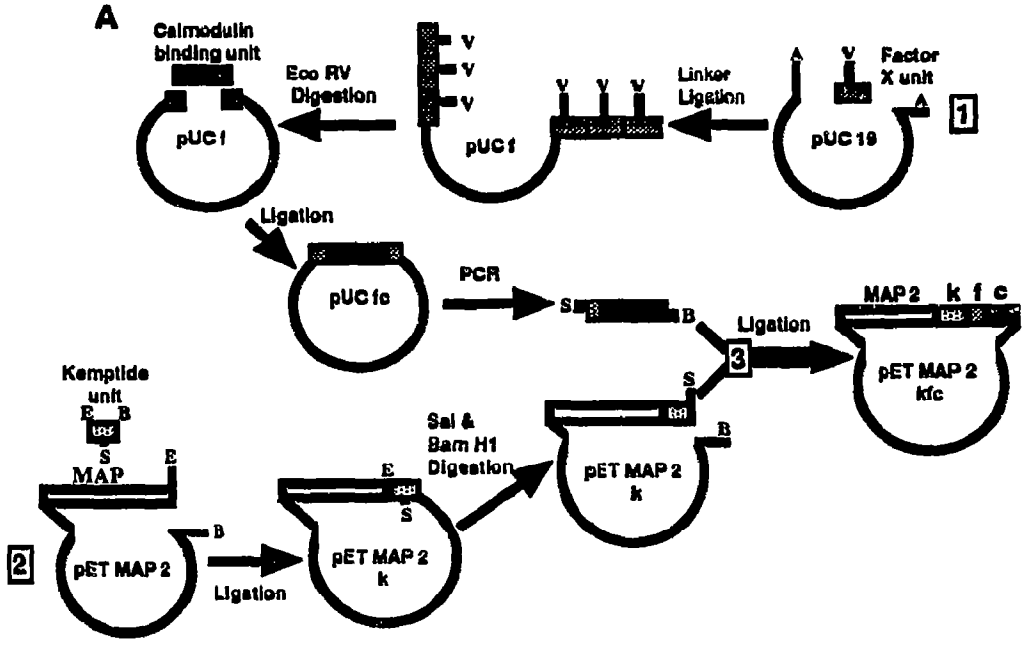

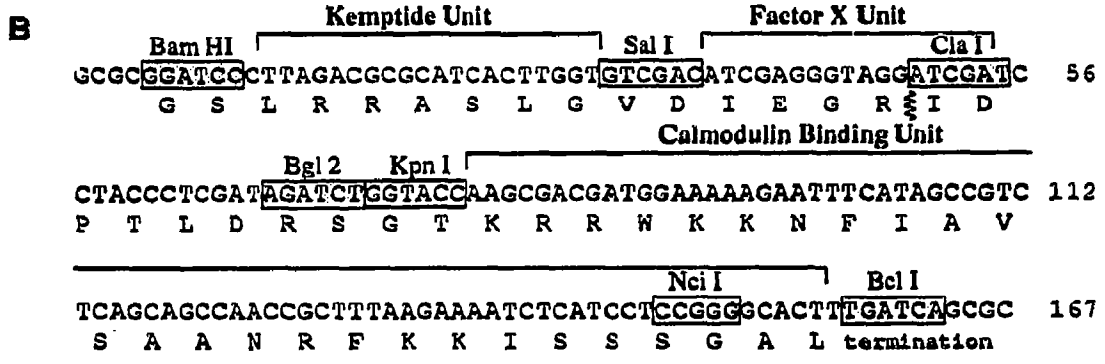

Fig. 1. Construction and sequence of the kfc cassette. (A) The kfc cassette was constructed in three stages. (1) The factor Xa/calmodulin-binding units were constructed in plasmid pUC 19. Complementary oligonucleotides encoding the factor Xa recognition sequence (-Ile-Glu-Gly-Arg-Ile-Asp-) were phosphorylated by polynucleotide kinase, annealed and ligated into *Sma*I-cut pUC 19. This produced concatamers of the factor Xa recognition sequences, which were cut back by digestion with *Eco*RV. Following this step, the linearized pUC f plasmid was reformed by inserting the calmodulin-binding unit (residues 577–602 of rabbit skeletal muscle myosin light chain kinase) between the factor Xa recognition sequences. Plasmid pUC fc was transformed into *E. coli* DH5α and plasmids isolated from individual colonies were sequenced to identify those which contained single copies of the factor Xa and calmodulin units that were ligated in frame with respect to each other. Once a correct construct was identified, it was used as a template to construct a 128 base pair DNA cassette with *Sal*I and *Bam*HI ends that encoded the factor Xa and calmodulin units. (2) A DNA cassette with *Sal*I and *Eco*RI ends encoding the kemptide sequence was inserted into pET MAP 2 (Δ1-154). The plasmid was propagated in *E. coli* DH5α and isolated. (3) The fc cassette was inserted into pET MAP 2 (Δ1-154)-k, which was cut with *Sal* and *Bam*HI, creating pET MAP 2 (Δ1-154)-kfc. Restriction enzyme sites are indicated: A, *Sam*I; B, *Bam*HI; E, *Eco*RI; S, *Sal*I; and V, *Eco*RV. (B) The nucleotide sequence of the kfc cassette is presented with the deduced amino acid sequence below. Boxed regions represent unique restriction sites and kemptide, factor Xa and calmodulin-binding sequences are highlighted. The cleavage site for factor Xa is marked ( ).

ligated to the MAP 2 (Δ1-154) coding region to create plasmid pET MAP 2 (Δ1-154)-k (Fig. 1A). Concomitantly, the factor Xa/calmodulin-binding units were constructed in pUC 19 creating plasmid pUC-fc (Fig. 1A). A 128 base pair factor Xa/calmodulin-binding unit from pUC-fc was amplified by the polymerase chain reaction (PCR) with *Sal*I and *Bam*HI ends and inserted into pET MAP 2 (Δ1-154)-k to create pET MAP 2 (Δ1-154)-kfc (Fig. 1A). To obtain a kfc DNA cassette that could be readily inserted into other bacterial expression vectors, the 167 base pair region from pUC MAP 2 (Δ1-154)-kfc was amplified by PCR with 5′ *Bam*HI and 3′ *Bcl*I sites at the ends. The sequence of this DNA cassette, presented in Fig. 1B, was designed for insertion into the *Bam*HI site of pET 11d.

2.2. *Purification of MAP 2 (Δ1-154) kfc*

2 l of LB broth containing 100 μg/ml ampicillin was inoculated with an overnight culture of *E. coli* transformed with pET MAP 2 (Δ1-154)-kfc. The cells were grown to an OD_{550} of 0.3 in a 37°C shaking incubator. Maximal MAP 2 (Δ1-154)-kfc expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the cells were grown for an additional 2.5 h to allow the accumulation of the recombinant protein. The culture was centrifuged at 8,000 rpm for 8 min at 4°C and resuspended in 25 ml of Buffer A (20 mM Tris, pH 7.0, 1 mM imidazole, 1 mM Mg-acetate, 0.1 mM $CaCl_2$, 10 mM β-mercaptoethanol, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM benzamidine). Bacterial lysis was achieved either by two passages

through a French press or by two cycles of freeze–thawing and sonication. Bacterial lysates were placed on ice and incubated with 40 μg/ml DNAse I for 1 h with gentle agitation. Soluble protein extracts were transferred to a 50 ml conical tube and mixed with 10–15 ml of calmodulin–agarose on a rotary mixer for 2 h at 4°C. After gentle centrifugation (1,000 rpm) to settle the affinity resin, unabsorbed soluble protein was decanted and the calmodulin–agarose was resuspended in 15 ml of Buffer A and poured into a 1.5 × 10 cm column. The column was washed with 10 vols. of high-salt buffer (Buffer A/1 M NaCl) to release non-specifically bound proteins, then re-equilibrated in wash buffer. MAP 2 (Δ1-154)-kfc was eluted from calmodulin–agarose with 20 ml elution buffer (20 mM Tris, pH 7.0, 1 mM imidazole, 1 mM Mg-acetate, 2 mM EGTA, 10 mM β-mercaptoethanol) and protein samples were collected in 1.5 ml fractions. The purity of each recombinant protein was assessed by SDS-polyacrylamide gel electrophoresis.

2.3. *Band shifting on non-denaturing electrophoresis gels*

The ability of MAP 2 (Δ1-154)-kfc to form complexes with RIIα was assessed by non-denaturing gel electrophoresis. The RIIα/MAP 2 (Δ1-154)-kfc complex migrates slower than either free RII or MAP 2 (Δ1-154)-kfc and could be easily distinguished. Polyacrylamide gels were prepared as described by Laemmli [14] with the omission of SDS in all reagents. Samples were incubated for 1 h at 20°C (23 μl total volume), diluted with 100 mM MOPS, pH 6.8, 40% glycerol, 0.014% Bromophenyl blue (7 μl) and loaded on the gel. Free- and complexed proteins were separated by electrophoresis on 6% (w/v) polyacrylamide gels at 45 mA for 4 h at 10°C. Protein bands were detected using Fast Stain (Zoion Research).

2.4. *Miscellaneous methods*

The nucleotide sequences of all constructs were analyzed as determined by the method of Sanger and colleagues [15]. Analysis of SDS-polyacrylamide gel-electrophoresis was performed by the method of Laemmli [14]. MAP 2 (Δ1-154)-kfc was phosphorylated as described by Scott and colleagues [16]. The solid-phase calmodulin-binding assay was performed as described by Glenny and Weber [17]. Factor Xa cleavage followed the protocol outlined in the Pierce instruction manual #32520/21. Recombinant RIIα was purified as described by Scott and colleagues [18].

3. RESULTS

A prominent protein band of M_r 26,000 was detected in soluble cell lysates of IPTG-induced *E. coli* cells containing pET MAP 2 (Δ1-154)-kfc (Fig. 2A, lane 1). MAP 2 (Δ1-154)-kfc expression was maximal at 2.5 h after IPTG induction, representing approximately 5–10% of the total soluble protein, as assessed by SDS polyacrylamide gel-electrophoresis (Fig. 2A, lane 1). Incubation with calmodulin–agarose specifically adsorbed MAP 2 (Δ1-154)-kfc. This single chromatography step removed most of the detectable MAP 2 (Δ1-154)-kfc from bacterial cell lysates (Fig. 2A, lane 2). Homogenous MAP 2 (Δ1-154)-kfc was eluted from calmodulin–agarose by washing in buffers containing 2 mM EGTA (Fig. 2A, lane 3). Approximately 15 mg of MAP 2 (Δ1-154)-kfc was isolated per liter of bacteria.

The kfc tail of the purified MAP 2 (Δ1-154)-kfc was tested for its ability to be phosphorylated, cleaved by factor Xa, and bound by calmodulin. Purified MAP 2 (Δ1-154)-kfc was phosphorylated by the C subunit of PKA (Fig. 2C), as was the MAP 2 (Δ1-154)-kfc in crude bacterial cell lysates (data not shown). Residues 1–154

Fig. 2. Purification and phosphorylation of MAP (Δ1-154)-kfc. (A) Protein samples taken at various stages of the MAP (Δ1-154)-kfc purification were analyzed by SDS gel electrophoresis on a 12% (w/v) polyacrylamide gel. Lane 1, crude bacterial lysate; lane 2, flow through from calmodulin-agarose column; lane 3, MAP 2 (Δ1-154)-kfc eluted from calmodulin-agarose. The molecular weight markers are indicated to the left of lane 1. (B) Purified MAP 2 (Δ1-154)-kfc was phosphorylated by the cAMP-dependent protein kinase with [^{32}P]ATP as described by Scott et al. [16]. Phospho MAP 2 (Δ1-154)-kfc was separated from free [^{32}P]ATP by SDS gel electrophoresis on a 12% (w/v) polyacrylamide gel. Lane 1 shows an autoradiograph of the gel.

of MAP 2 do not contain any PKA phosphorylation sites [19,20] and cannot be phosphorylated by the kinase. These results demonstrate that the kemptide sequence (Fig. 1B) within the kfc tail allows the specific incorporation of a radioactive tag onto the recombinant protein.

MAP 2 (Δ1-154)-kfc and two other expressed kfc fusion proteins can be detected by solid-phase calmodulin-binding assay (Fig. 3). This provides a highly sensitive and non-radioactive alternative method to detect the expression of the fusion protein.

The calmodulin-binding sequence of kfc fusion proteins can be removed by proteolysis with factor Xa enzyme. The calmodulin-binding seuqence of MAP 2 (Δ1-154)-kfc was almost completely removed by factor Xa after 60 min incubation at room temperature, while the same sequence attached to the type II regulatory subunit of the cAMP-dependent protein kinase (RIIα) was partially resistant to proteolysis (Fig. 4). The efficiency of factor Xa cleavage appears to be dependent, in part, upon the primary structure of the kfc fusion protein.

Biological activity of MAP 2 (Δ1-154)-kfc was assayed by its ability to associate with RIIα as measured by band-shift analysis (Fig. 5). MAP 2 (Δ1-154)-kfc retained full biological activity when fused to the kfc affin-

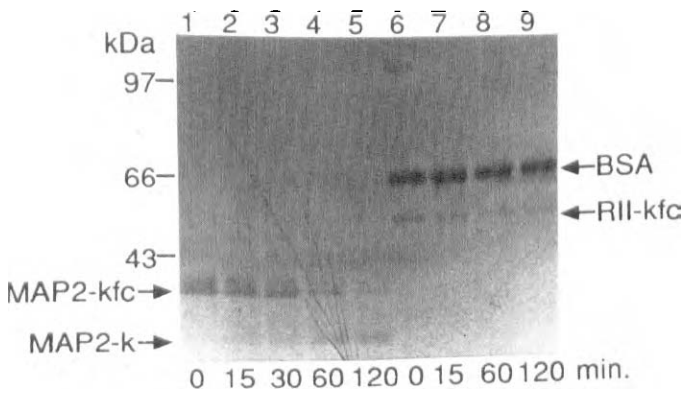

Fig. 3. Factor Xa cleavage of the calmodulin-binding unit. MAP 2 (Δ1-154)-kfc (10 μg) or RII-kfc (10 μg) were incubated with purified bovine factor Xa (0.5 μg = 0.09 U) for the indicated times (0–120 min) in a total volume of 110 μl. Factor Xa digestion was performed using the buffers and experimental conditions described by Nagai and Thogersen [11]. At the specified times (indicated below the lanes) 20 μl aliquots of reaction mixture were removed and the efficiency of factor Xa cleavage was assessed by SDS gel electrophoresis on a 12% (w/v) polyacrylamide gel. The migration positions of MAP 2 (Δ1-154)-kfc and MAP 2 (Δ1-154)-k and RIIa-kfc are indicated. Lanes 1–5, MAP 2 (Δ1-154)-kfc; lanes 6–8 RIIα-kfc; molecular weight markers are indicated. BSA (1 mg/ml) was added to RII-kfc reactions to stabilize the protein.

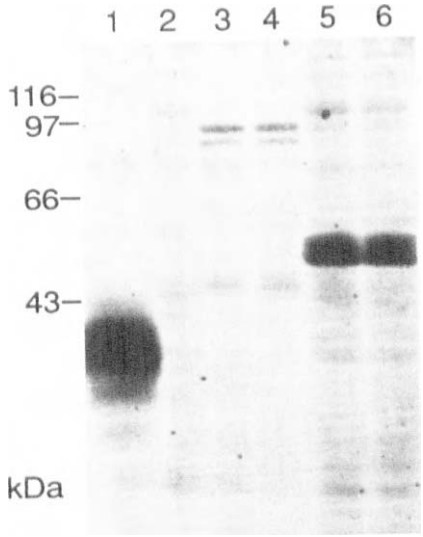

Fig. 4. Detection of kfc fusion proteins by solid-phase calmodulin-binding assay. Soluble protein extracts (40 μg) from bacteria expressing three kfc-fusion proteins, MAP 2 (Δ1-154), and two RII-anchoring proteins, Ht 21 [28] and Ht 31 [29], were separated by SDS gel electrophoresis on a 12% (w/v) polyacrylamide gel and electro-transferred to an immobilon filter. Calmodulin-binding proteins were detected by the solid-phase binding assay described by Glenney and Weber [17]. Lane 1, MAP 2 (Δ1-154)-kfc; lane 2, uninduced bacterial extract; lanes 3 and 4, Ht 21-kfc from two different clones; lanes 5 and 6, Ht 31-kfc from two different clones; molecular weight markers are indicated.

ity tail. A putative amphipathic helix is responsible for association with RIIα [19,20]. It is located within a 31-amino acid segment (residues 82–113) on MAP 2 (Δ1-154)-kfc.

4. DISCUSSION

In this report we describe the construction and characterization of a DNA cassette, kfc, which encodes three distinct peptide units designed to expedite the detection and purification of recombinant proteins. The kfc fusion system has so far been used to successfully purify 8 different proteins. An advantage of this system is that kfc fusion proteins can be rapidly purified to near homogeneity in a single chromatography step on calmodulin–agarose; a typical time-course from inoculation of liquid media to isolation of pure protein is 48 h. We believe that the kfc cassette is a versatile reagent with a potential for wide application in the purification of recombinant proteins.

The kfc DNA cassette can be inserted into a variety of commercially available bacterial expression vectors. Variants of the kfc cassette with unique restriction sites at the 5′ and 3′ ends can be produced by PCR and specifically tailored for insertion into the expression vector of choice. For the purposes of this report we have documented the expression and purification of an RII-binding fragment of MAP 2 from pET 11d, a carrier vector which utilizes a T7 polymerase for expression [21]. However, we have also successfully produced kfc proteins in pUC-based vectors (R.E. Stofko-Hahn and J.D. Scott, unpublished observation).

The kemptide sequence (Leu-Arg-Arg-Ala-Ser-Leu-Gly-) is a consensus PKA phosphorylation site adapted from rat liver pyruvate kinase which forms the first unit of the kfc peptide tail [22]. The serine in this sequence is efficiently phosphorylated by the C subunit of PKA with K_m values of 4–16 μM [9]. Fusion of the kemptide sequence to a recombinant protein allows quantitative incorporation of ^{32}P at a single site and permits detection by autoradiography, even in crude bacterial lysates. Similarly, the calmodulin-binding unit (Lys-Arg-Arg-Trp-Lys-Lys-Asn-Phe-Ile-Ala-Val-Ser-Ala-Ala-Asn-Arg-Phe-Lys-Lys-Ile-Ser-Ser-Ser-Gly-Ala-Leu) derived from the carboxyl-terminus of rabbit skeletal muscle myosin light chain kinase [13,23] can serve as a useful marker to detect the expression of kfc proteins. Biotinylated calmodulin retains its ability to bind to immobilized kfc fusion proteins and is detectable using streptavidin-conjugated alkaline phosphatase [24]. This provides a highly sensitive non-radioactive technique which can detect as little as 10 ng of recombinant protein (Fig. 4).

Removal of the calmodulin-binding sequence can be achieved by treatment with the coagulation factor Xa. Cleavage occurs (Fig. 1B) after the recognition sequence Ile-Glu-Gly-Arg- [10]. Previous studies have suggested that the factor Xa cleavage rate is also dependent upon the sequences downstream of the cleavage site [11,25]. However, our evidence suggests that factor Xa activity may also be effected, in part, by the structure of the

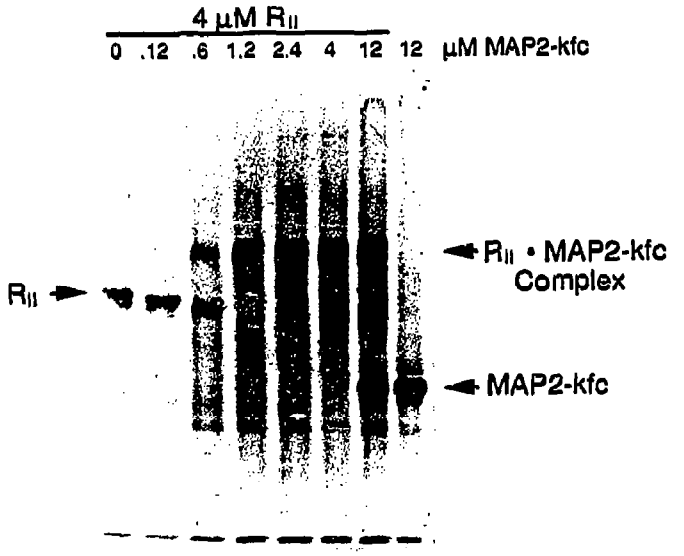

Fig. 5. Band-shift analysis of MAP 2 (Δ1-154)-kfc/RII interaction. RIIα (4 μM) (in lanes under the line) and various concentrations of MAP 2 (Δ1-154)-kfc (0–12 μM) were incubated at room temperature as described in section 2.3. and separated by non-denaturing gel electrophoresis. Free RIIα, MAP 2 (Δ1-154)-kfc and the RII/MAP 2 (Δ1-154)-kfc complex are indicated. The unlabeled band that appears only at the higher concentrations of MAP2-kfc and migrates between the free MAP2-kfc and RII/MAP2-kfc complex may represent a weakly binding complex of MAP2-kfc and either RII monomer or a proteolytic fragment of RII. The concentration of MAP 2 (Δ1-154)-kfc is indicated above each lane.

recombinant protein upstream of the cleavage site. This hypothesis is supported by evidence presented in Fig. 3 showing that MAP 2 (Δ1-154)-kfc is a better substrate for factor Xa than RIIα-kfc. However, as is the case for MAP 2 (Δ1-154), the kfc tail has little or no adverse effect upon the biological activity of the molecule (Fig. 5).

In some cases, expression of kfc fusion proteins can promote the formation of inclusion bodies. Although not the case for MAP 2 (1-154)-kfc, our general experience has been that proteins of approximately 100 amino acids or less are predominantly detected in the particulate fraction when expressed with the kfc tail. The ratio of soluble vs. particulate kfc protein, as monitored by the solid-phase calmodulin-binding assay, is different for each of the recombinant proteins we have expressed so far. Only in one case, the expression of a 107 amino acid fragment from the RII-anchoring protein, Ht 21, was all of the protein present in inclusion bodies. Formation of inclusion bodies may be decreased if bacteria are induced at 25–30°C or in the presence of 'compatible osmolytes' such as glycyl betaine, which are believed to cause preferential hydration and stabilization of the recombinant protein structure [26,27].

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