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Received 5 September 1994

Neuronal aspects of cytosolic chaperonin complexes: structures implicated in the production of functional cytoskeletal proteins

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Introduction

Three major fibrillar elements constitute the axoplasmic cytoskeleton of neurons: microtubules (MTs), assembled from α- and β-tubulin dimers, microfilaments (MFs), made of actin, and neurofilaments (NFs), the neuron-specific intermediate-sized filaments composed in mammals from the fibrous NF-H, NF-M and NF-L 'triplet' polypeptides [1]. Like other cytoplasmic proteins these cytoskeletal components are all thought to be synthesized on ribosomes restricted to the cell body, being then delivered to the axon and synapse by slow axonal transport. Knowledge about how these various components are synthesized, assembled and distributed in neurons, however, still contains significant gaps. First, the sites of polymer assembly and forms in which cytoskeletal proteins move in axoplasm, i.e. whether as polymer, free assembly-competent subunits or some admixture, remain subjects for hot debate [2]. Moreover, it is now emerging that the correct folding of proteins generally *in vivo*, long assumed from *in vitro* re-folding studies to be spontaneous and dictated solely by amino acid sequence [3], is now viewed as likely to require a set of catalytic proteins, collectively termed molecular chaperones, to prevent inappropriate folding interactions leading to protein aggregation at the high concentrations of protein present in neuronal perikarya.

We first became interested in molecular chaperones and their link with the biogenesis of the neuronal cytoskeleton during studies of proteins binding to, and then released by ATP from, an affinity column to which was attached a recombinant protein representing part of the major, repetitive phosphorylation domain from the mouse NF-H component [4]. Upon electron microscopy of the material liberated from these columns by ATP, following loading with a spinal cord extract we noted hollow, ring-shaped particles having the characteristic morphology of a particular type of molecular chaperone, the chaperonin complex (reviewed in [3,5–8]). This identification was confirmed using a monoclonal antibody (mAb) to the mouse t-complex polypeptide 1 (TCP1), then recently implicated as a component of a previously unrecognized cytosolic chaperonin [9,10]. As other reports appeared implicating this chaperonin more directly in the folding of cytoskeletal proteins, especially of tubulins and actins [11–20], we became interested in determining whether there might be any specifically neuronal aspects to cytoplasmic chaperonin structure, composition, specificity or subcellular distribution. In view of the extensive cytoskeletal networks and abundance of actin and, especially, tubulin in nerve cells, cytoplasmic chaperonin activity might be expected to play an important role in the normal turnover of cytoskeletal components in mature neurons. Moreover, they would be especially important during neuronal differentiation and neurite outgrowth, when much new cytoskeletal protein synthesis and polymer re-modelling occurs. We describe at the end of this paper some recent findings made using a cell line (ND7/23 [21]) to model the early events associated with cytoskeletal elaboration during neurite outgrowth.

Abbreviations used: CCT, chaperonin containing TCP1-related components; i.e.f., isoelectric focusing; mAb, monoclonal antibody; MF, microfilament; MT, microtubule; NF, neurofilament; TCP1, t-complex polypeptide 1.

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Cytosolic chaperonins and the biogenesis of actin and tubulin proteins

Tubulin was first purified from brain, a tissue where it is particularly abundant, being the main component of the MT 'tracks' required for fast axonal transport and, indirectly, exocytotic neurotransmitter release. Tubulin is, however, a ubiquitous eukaryotic cell component required not least importantly for spindle MTs on which chromosomes move during mitosis. Likewise actin, first purified from muscle, is also generally required for eukaryotic cytokinesis and is arguably the most widely distributed and highly conserved cytoskeletal protein of all. With the importance of these two cytoskeletal components in mind, it had long been puzzling that neither protein could be re-folded alone in the test tube under any condition tested. Nor, despite abundant polypeptide production, could either of them be recovered in an assembly-competent state from bacteria engineered to express them. In hindsight, it is clear that both protein types require chaperone action during, or soon after, their synthesis and it follows therefore that this (or these) chaperone(s) must, like these substrates, also be ubiquitous in eukaryotic cytoplasm and absent from bacteria. The first clues to chaperone involvement in cytoskeletal protein production arose from observations that correctly folded (polymerization-competent) actin monomer or tubulin dimer can, in fact, be produced *in vitro*, if a mammalian reticulocyte lysate translation system is used. Closer analysis of the reticulocyte lysate itself was particularly informative: on the one hand it was reported that during translation tubulin enters a high-molecular-mass (around 900 000 Da) complex, prior to its ATP-dependent release [11]. In a second report, appearing also in 1992, bacterially expressed, denatured actin was found to re-fold under the influence of particles purified from reticulocyte lysates having the morphology of chaperonins, once again to generate a polymerization-competent product and in an ATP-dependent manner [12]. In both cases, the protein TCP1, suggested earlier as a component of an until then elusive eukaryotic cytosolic chaperonin [9,22], was implicated as a constituent of the complex involved in folding activity. The supposition that TCP1 is, indeed, a cytosolic chaperonin component was confirmed that same year [10] and the complex was later reported to bind actin and tubulin as its major substrates *in vivo* [16]. Because of the present reliance upon antibodies to TCP1 to characterize the various eukaryotic cytosolic chaperonin complexes described so far (TCP1 being a conserved component thereof) we adopt here the terminology suggested by Kubota et al. [23] for these particles, namely chaperonins-containing TCP1-related subunits, conveniently abbreviated to CCT.

Chaperonins

The term chaperonin is now widely used to distinguish this particular structurally and functionally related group of molecular chaperones from other types. The main distinguishing features of chaperonins are as follows: first, their oligomeric composition from polypeptide subunits of size around 60 kDa (hence their alternate classification as cpn60-type molecular chaperones); and, secondly, their characteristic particulate morphology: in all cases cpn60 components assemble into two rings containing 7–9 subunits each (numbers being fixed for the chaperonin from a particular source, but different among the various chaperonin types). These rings stack face-to-face into a doubly toroidal, cylindrical particle with external diameter around 16 nm and a central hollow [8]. It is within this cavity that folding substrates are purported to fit [18,24,25], doing so under the regulation of ATP-dependent conformational changes in the individual subunits making up the chaperonin rings [12,26]. Chaperonins have now been identified in both major classes of bacteria, in mitochondria and in chloroplasts, and, finally, now in the eukaryotic cytoplasm. The polypeptides composing CCTs more closely resemble the chaperonin (cpn60) components of archaebacteria [9,10,23] than those of either the mitochondrial or chloroplast chaperonins which, in turn, are more related to the eubacterial cpn60, i.e. GroEL, reflecting the probable endosymbiotic origin proposed for these eukaryotic organelles. Chaperonin presence in the nucleus is still unresolved: whether 'cytosolic' chaperonins enter, as their substrate actin does, is still not clear.

Structure, composition and function of eukaryotic cytoplasmic chaperonins

While sharing with other reported types of chaperonin the double toroidal structure and certain features in the primary sequences of their subunit proteins, including probable ATP-binding and hydrolytic domains, CCTs differ in not yet having been demonstrated to associate with small (cpn10) subunits. For the other chaperonin types these cpn10 subunits are assembled as a seven-membered ring which can bind to either end of the cpn60 double-toroid particle and in some cases are required for folding certain protein substrates successfully [7].

CCTs are further unique in that their subunit proteins are not induced at significantly higher expression levels during cellular heat shock [10]. The most important difference, however, is the heterooligomeric composition of CCTs in a variety of organisms from human through yeast to oat plants [10,13,15,23,27], as opposed to being composed of just one subunit type or two , like the chaperonins of mitochondria, chloroplasts and bacteria.

As already mentioned, by virtue of sequence similarity with then known chaperonin components [9,22], the first candidate component of the still, at that time, elusive cytoplasmic chaperonin was the TCP1 polypeptide, originally characterized in mouse testes [28] where it is particularly abundant. Lewis et al. [10] verified not only that TCP1 protein is assembled into particles with the chaperonin morphology, but also that it had a cytoplasmic distribution in cells. They further showed that some six to eight other polypeptides of similar size were co-immunoprecipitated using TCP1-specific antibodies after just 10 min of pulse-chase labelling. This composition was soon confirmed by others, including ourselves [4], with limited microsequence data obtained for certain of the individually purified components [13,15] establishing the close relationship of each with the known TCP1 sequence [29]. This was firmly established upon cloning of six more entire sequences for the cytosolic chaperonin components, denoted CCTβ, CCTδ, CCTε, CCTγ, CCTη and CCTζ [23], TCP1 now being denoted CCTα. Moreover, comparison with sequence data for non-mammalian CCT subunits has established their evolutionary conservation and suggests that each and every CCT polypeptide may be a necessary component of the CCT complex and that the CCT itself has a vital function throughout the eukaryotic kingdom. These conclusions are supported by the lethality of deleting, in yeast, the genes encoding the orthologues of CCTα [30] and CCTβ [31].

The heteromeric composition of CCTs now seems beyond doubt. It remains unclear, however, whether there is in all tissues just a single CCT of invariant stoichiometric composition and conserved structure and function. This view, put forward by Rommelaere and colleagues [15], is in keeping with immunoprecipitation data [10] and failure of many researchers to resolve apparently different CCT populations using diverse non-denaturing chromatographic separation procedures. However, it does not accord well with the identification of an apparently testis-specific CCT subunit absent from brain tissue [4], and it is not easy to understand how a single structure containing either eight- or nine-membered rings could be composed from the identified range of CCT subunits, numbers and comparative expression levels of which vary subtly among cell types. Kubota et al. [23] have instead proposed that CCT particles might be composed of variable contributing subunits, depending on tissue type and according to which of the CCT subunits are actually expressed by the cells therein. Further, it seems possible that CCT composition might vary within individual cells according to particular functional folding (substrate) requirements. In this respect, an interesting study was reported last year [32], though not yet published in full, which described immunoprecipitation from brain extracts of a homomeric 20S particle (the expected size of a chaperonin complex) using an antiserum to TCP1, i.e. it gave evidence of a CCT composed solely from the CCTα component. It also reported, incidentally, that TCP1 was located in nuclei in addition to the cytoplasm.

So far, little has been said about the stage at which CCTs are thought to participate in the folding of proteins. A seminal paper just published [33], at the time of writing, has greatly clarified the picture: using the rabbit reticulocyte lysate system to study the folding of newly synthesized luciferase, it was first confirmed, as suspected, that CCTs cooperate with other, non-chaperonin types of molecular chaperones, namely the heat-shock-related proteins hsc(hsp)70 and hsp40. It was further shown that none of these chaperones (hsp40, hsc/hsp70 or the heterooligomeric CCT) are liberated from nascent polypeptides undergoing translation (or from ribosomes) unless both a ribosomal release signal (stop codon or the drug puromycin) and ATP were both added. These findings indicate close interactions between these three classes of molecular chaperone and, moreover, their co-translational action. Furthermore, the findings strongly indicate that in eukaryotes CCTs participate in a folding pathway similar, if not identical, to that proposed in bacteria, involving the chaperone protein analogues DnaJ (hsp40), DnaK (hsp70) and GroEL/ES (cpn60/10) [7]. It is important to point out, however, that a second study [34], suggests that co-translational folding of single polypeptides is probably not the only role for CCTs in cells. Its results strongly suggest that during new synthesis of viral capsid polypeptides in a cell-free transcription-linked system CCTs are involved in the oligomerization step producing the final mature assembled capsid product. This finding is especially interesting in respect of neuronal cytoskeletal pro-

tein biogenesis, where it may be reasonable to propose that CCTs could play a role in assembling their substrate proteins into oligomers, or else maintaining their folded integrity, at sites long distant from the perikaryally delimited ribosomes. If this were the case, then CCTs should be found in axoplasm and dendrites in addition to the 'translational cytoplasm' of the neuronal cell body. It was to test this idea that we embarked upon experiments with differentiating ND7/3 cells, described in the next section.

CCTs in a differentiating 'neuronal' cell line

We had shown [4] that CCTs isolated from rat and guinea-pig brain tissue residually contain, and rebind, unfolded tubulins and display essentially the same subunits, defined by 'spot profile' patterns in two-dimensional [isoelectric focusing (i.e.f.)/SDS/PAGE] gel patterns, as CCTs isolated from testis of the same animals, excepting the testis-specific component mentioned earlier. This latter finding was rather disappointing because we had hoped to find more convincing evidence for heterogeneity of CCTs from brain compared with testis, it having been shown that a human orthologue of the bacterial DnaJ molecular chaperone is brain-specific [35]. Nonetheless, since neurons elaborate a very highly asymmetric and regionally specialized cytoplasm, they may still serve in establishing whether CCTs, like ribosomes, are limited to perikarya or else enter axons. Moreover, since the bulk brain tissue we used before is a complex admixture of neuronal and non-neuronal cell types, and subregions thereof, there was little chance of selectively purifying axonal CCTs even if they were present. Thus we have now chosen to study a clonal cell line with neuronal properties and, moreover, one which can be differentiated in low-serum, nerve growth factor and dibutyryl cyclic AMP conditions to form one or two axon-like neurites. The ND7/23 cell line chosen (the kind gift of Dr. John Wood, Sandoz Medical Research Institute, London) is a neonatal rat dorsal root ganglion cell × mouse neuroblastoma hybrid [21] and can be cultured in numbers amenable both to biochemical and immunocytochemical observations. Our studies, described only in outline here, are to be published in full elsewhere.

A necessary preliminary was to purify by established procedures [4,10] the CCTs from undifferentiated ND7/23 cells and determine their subunit composition and cross-reactivities with antibodies generated to CCT components from other sources. The two-dimensional gel patterns of the pure ND7/23 cell CCTs were very similar to the 'definitive' mouse CCT profile published by Kubota et al. [23], and an antiserum generated to rat testis CCTs (A. Roobol, unpublished work) collectively recognized three of the ND7/23 CCT components, corresponding to CCTβ, CCTε and CCTγ, but not CCTα (TCP1), which instead could be detected with mAbs from Keith Willison [10]. Upon differentiation, this two-dimensional gel pattern was largely unchanged, with the exception that CCTα was unexpectedly diminished and so were both tubulin polypeptides (α and β chains detected with mAbs donated by Professor Keith Gull, University of Manchester). In immunoblots of standard one-dimensional SDS/PAGE-separated proteins that had been extracted directly from differentiated ND7/23 cells using a harsh denaturant (SDS) tubulins were, on the other hand, actually both found to be slightly increased upon ND7/23 cell differentiation and the CCTα levels unchanged. This discrepancy appears explicable when it is appreciated that in extracting cell proteins for two-dimensional gels denaturant was not used, being avoided because prolonged exposure to urea (needed for i.e.f.) can result in multiple spotting of CCT subunits, presumably due to their carbamylation sensitivity. Subjecting the same culture dishes to subsequent denaturing extraction in SDS extracted additional proteins which had been left behind after the two-dimensional gel sampling and this material was found to be correspondingly enriched in tubulins and CCTα (around 50% of the total originally present), but not in any of the other quantifiable CCT subunits (CCTβ, CCTε and CCTγ). This observation indicates that CCTα and tubulins were, for some reason (possibly their functional interaction), partially resistant to extraction under non-denaturing extraction of cell proteins. Sucrose-gradient fractionation of CCTs from differentiated ND7/23 cells, performed after optimizing conditions for extracting all cellular CCT components, revealed all components to be in particulate form, migrating primarily as a peak with mass around 900 kDa. However, minor amounts of all the detectable CCT components (CCTα and CCTβ, CCTε and CCTγ) were also detected further down in the gradient (i.e. as larger or denser structures, that had also been detected in preparations from undifferentiated ND7/23 cells). There were no CCT subunits detectable at the top of the gradient, where free monomers would have fractionated, in preparations from either differentiated or undifferentiated ND7/23 cells.

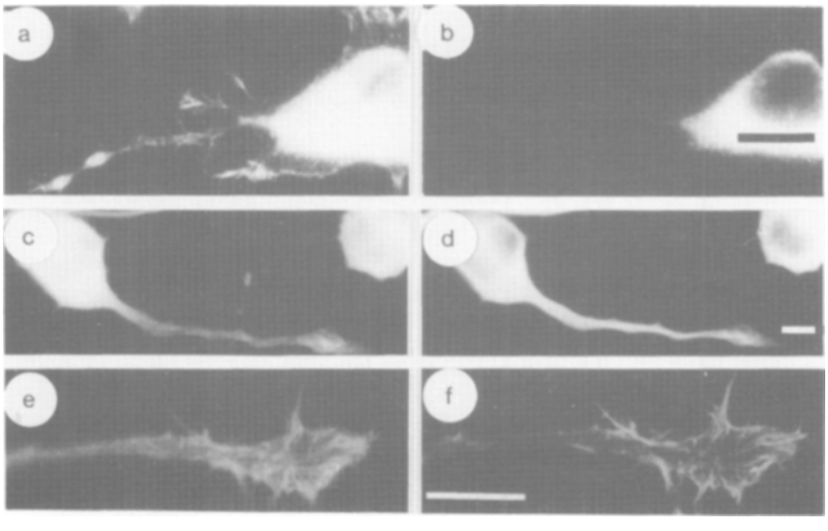
The results from immunofluorescent localization studies of CCTs in differentiated ND7/23 cells were the most revealing (Figure 1): staining with mAbs specific for TCP1 (CCTα) revealed a strongly neuritic fluorescence in addition to the expected perikaryal signal (Figure 1a). Affinity-purified antiserum recognizing CCTβ, CCTε and CCTγ, however, gave strong fluoresence only of the cell bodies, being barely detectable in neurites, although not altogether excluded (Figure 1b). Indeed, the staining pattern and distribution for these other CCT subunits was not unlike that (not shown) of a mAb to ribosomal S6 protein (donated by Dr. Chris Proud, University of Bristol), confirming the expected largely perikaryal restriction of ribosomes in newly differentiating ND7/23 cells. Antibodies to tubulin and actin gave more interesting patterns still with respect to the corresponding locations revealed for CCT proteins in double immunofluorescent staining. As expected, both cytoskeletal proteins were distributed all along the neurites (Figures 1d and 1f) and actin was recognized intensely lining the inner membrane faces of neurites and growth cones, and also found within filopodial microspikes extending from their edges (Figure 1f). CCTα staining patterns appeared to largely mirror the subplasmalemmal actin staining (i.e. was co-localized) although not, interestingly, to quite the same extent, the actin staining within the filopodial processes (compare panels e and f in Figure 1). Actin exists in two distinct forms, globular or G-actin (the unpolymerized state) and filamentous, or F-actin (the MF form). MFs are enriched inside growth cone filopodia [36], while the G-form of actin is enriched at cellular sites undergoing dynamic assembly of MFs, such as the cortical cytoplasm beneath plasma membranes.

Taken together, our results seem to strongly suggest several very interesting conclusions about neuronal CCTs. First, CCTα enters neurites in a particulate (CCT complex) form during early neuritogenesis. Thus CCTs can be distributed away from the ribosomal protein synthetic machinery of the neuron. Secondly, CCTα enters neurites to an obviously greater extent than certain other CCT components, and yet both are particulate based on sucrose-gradient analysis. This argues for heterogeneity of CCT composition (and, possibly, function) within single cells. Lastly, CCTα co-localizes with one of its known cytoskeletal protein-folding substrates, namely actin (and possibly also with tubulin based on the biochemical extraction evidence described above) and, moreover, seemingly just the unpolymerized form. This suggests a function for CCTs containing, or perhaps enriched for, the CCTα (TCP1) subunit in chaperoning (main-

Figure 1

Double immunofluorescent labelling of CCT subunits and cytoskeletal proteins in ND7/23 cells

ND7/23 cells differentiated in tissue culture for 3 days using low-serum, nerve growth factor and dibutyryl cyclic AMP [37] were fixed and doubly stained with a mAb (84a, the kind gift of Keith Willison [10]) to CCTα (**a–c**) plus an affinity-purified antiserum to rat testis CCTs recognizing CCTβ, CCTε and CCTγ (**b**) or a mAb (TAT-1 [38], the gift of Keith Gull) to α-tubulin (**d**), or a mAb to β-actin [39] (**f**), purchased from Sigma. Each bar indicates 10 μm.



taining the native structure of) unassembled (G-) actin and even, perhaps, an additional role in its incorporation into MFs.

Concluding remarks

Present understanding about the biogenesis of the neuronal cytoskeleton places heavy reliance upon the concept of self-assembly by its various components. In the CCT field, on the other hand, the emphasis so far has been concentrated largely upon their roles in the initial production (new synthesis) of proteins, largely because CCTs are not thought to be induced by cell shock and so not implicated in repairing damaged (unfolded) proteins. Investigation of potential roles for CCTs in maintaining the native folding state of already synthesized proteins and also, to a lesser extent, their oligomerization, has received rather scant attention. The asymmetry of the neuronal cytoplasm and its highly developed, specialized and comparatively well-understood cytoskeleton appear to make this an excellent arena in which to discover new aspects of CCT function, distribution, composition and interactions. Moreover, such studies should further extend and inform our present knowledge of the neuronal cytoskeleton.

We thank our colleagues Nandini Hayes, Fiona Holmes and, especially, Anthony Baines for help, advice and discussion and acknowledge their participation in the studies of ND7/23 cells. All work on CCTs has been greatly facilitated by mAbs to TCP1 donated by Dr. Keith Willison (Chester Beatty Laboratories, London) and by his generous advice and free communication of recent findings and ideas about chaperonins. We are also grateful to our other collaborators (Keith Gull, Chris Proud and John Wood) for contributing other antibodies, or cell lines, as mentioned in the text. Studies were supported at various stages by grants from the NIH, SERC and Wellcome Foundation.

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Received 5 September 1994

Post-translational modifications of microtubule- and growth-associated proteins in nerve regeneration and neuropathy

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Introduction

Post-translational modifications of cytoskeletal proteins occur in cultured neuronal cells in response to stimuli that induce neurite outgrowth [1–3]. Some of these changes also occur in mammalian peripheral nerve during regeneration following axotomy, e.g. tyrosination of α-tubulin [4] and phosphorylation of β-tubulin [5]. Phosphorylation of the microtubule-associated protein MAP-2 also shows enhanced phosphorylation during neurite extension in culture [3,6]. A number of protein kinases can phosphorylate microtubule and microtubule-associated proteins (MAPs), including Ca^{2+}/calmodulin-dependent kinase and cyclic AMP-dependent kinase [7]. To determine whether these changes also occur in regenerating peripheral nerve, we examined the activity of a number of protein kinases in the sciatic nerve of the rat against purified microtubule proteins and MAPs.

The growth-associated protein GAP-43 is also phosphorylated in response to factors responsible for neurite outgrowth [8], although the process of phosphorylation of GAP-43 in peripheral nerve is not understood. Experimental diabetes is associated with an impairment of peripheral nerve regeneration [9], part of which may be due to altered processing or transport of GAP-43 [10]. We therefore also examined the protein kinase activity of peripheral nerve directed against GAP-43 both under normal conditions and during experimental diabetes.

Abbreviation used: MAPs, microtubule-associated proteins.

Methods

All operative procedures were carried out according to guidelines set out in U.K. Home Office Licence PPL 40/00230.

Female rats were anaesthetized with diazepam, fentanyl and fluanisone and one sciatic nerve was crushed with silk thread. The contralateral nerve served as an internal control. Five days later, nerves and dorsal root ganglia were removed and nerves were divided into regions proximal and distal to the site of the crush. In some experiments rats had been rendered diabetic four weeks previously by a single intraperitoneal injection of streptozotocin. Rats with a blood glucose level > 15 mM were considered to be diabetic. Tubulin and MAPs were prepared from pig brain by differential centrifugation of GTP-polymerized microtubules, followed by phosphocellulose chromatography. GAP-43 was purified from neonatal rat brain [11]. Purity was confirmed by SDS/PAGE. Sciatic nerve pieces were homogenized in 100 mM Mes buffer, pH 6.4, or 20 mM Tris buffer, pH 7.5, containing 1 mM EGTA and protease inhibitors. Homogenates were centrifuged at 100 000 ***g*** for 1 h to separate soluble and particulate fractions. The protein kinase activity of sciatic nerve fractions was assayed by incubation of fractions with or without either heat-stable MAPs, purified tubulin or GAP-43, in the presence of [γ-^{32}P]ATP with (to stimulate specific kinases) 10 mM $MgCl_2$, 0.25 mM $CaCl_2$ and 50 μM calmodulin, or 0.75 mM $CaCl_2$ alone, or 10 μM cyclic AMP for 10 min at 37°C. Samples also contained, as appropriate, 1 μM okadaic acid, 20 mM NaF, 2 μM calphostin C or