Distinct signalling particles containing ERK/MEK and B-Raf in PC12 cells

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Although several multiprotein complexes containing MAPKs (mitogen-activated protein kinases) have been identified using overexpression of kinases and scaffold proteins, the components of the complexes and their physical properties at endogenous expression levels have not been defined. We characterized a large protein complex containing a nerve-growth-factor-activated ERK (extracellular-signal-regulated kinase) and MEK (MAPK/ERK kinase) in rat pheochromocytoma (PC12) cells. This protein complex fractionated into a high-speed pellet and was resistant to non-ionic detergent treatments that solubilized membranes. Disruption of protein–protein interactions by treatment with high salt was required to facilitate immunoprecipitation of active ERK1 and co-precipitation of MEK1. Microtubule fragments were also present in the detergent-resistant high-speed pellet, and some kinases were bound to them, especially ERK1b (an alternatively spliced isoform of ERK1), which showed a strong preference

for binding microtubules. The large protein complex containing ERK1 and MEK1 was resolved by velocity sedimentation from fragments of microtubules; however, it did not contain other scaffolding components known to bind ERK and MEK. B-Raf was also present in a distinct detergent-resistant, microtubule-independent protein complex slightly larger than that containing ERK and MEK. We conclude that there are two independent nerve growth factor-regulated ‘signalling particles’ with an estimated size of 60–75 S, one containing ERK1 and MEK1 and the other containing B-Raf. These signalling particles may have a role in the temporal and spatial regulation of kinase activity inside cells.

Key words: extracellular-signal-regulated kinase (ERK), mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK), nerve growth factor (NGF), PC12 cell, Raf, scaffold.

INTRODUCTION

MAPK (mitogen-activated protein kinase) cascades must be controlled at many levels. Components of several different kinase cascades interact promiscuously *in vitro*, yet transduce very specific signals and responses *in vivo*, which means that their fidelity must be appropriately directed to convey their individual signals. The efficiency with which signals are transferred, i.e. the fecundity of the signal, must also be controlled. The longevity or duration of the signal may also be used to dictate specific responses. Finally, the intracellular location of signalling from MAPK cascades may be regulated. Models to explain the regulation of MAPK modules now include scaffold proteins, which bind two or more signalling components [1]. It is now known that different scaffold proteins bring together components of distinct signalling pathways to control their signalling fidelity, fecundity, longevity and location.

The prototype scaffold is yeast Ste5, which binds yeast MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase] kinase (Ste11), MEK (Ste7) and MAPK (Fus3 or Kss1) as well as Gβ [2–4]. In recent years, the number of putative mammalian analogues of Ste5 has blossomed [1]. Considering only the proteins known to bind to the MAPK, ERK1, these include MP-1 (MEK partner 1) and Ksr (kinase suppressor of Ras) [5]. Immunofluorescence studies reveal that these two different scaffolds organize kinase modules in different intracellular locations, which allows distinct compartmentalization of signal transduction. MP-1, through binding to p14, targets MEK and ERK to the

late endosome [6,7]. Ksr, in contrast, brings components of this kinase module to the plasma membrane [8].

NGF (nerve growth factor) activates a kinase cascade that activates ERK1 for a very long time (hours), promotes survival and enhances differentiation of nerve cells [9]. The receptor tyrosine kinase for NGF, gp140^{TrkA} (where TrkA is a receptor tyrosine kinase for NGF, a product of the *trk* oncogene) suppresses programmed cell death and activates the expression of the genes associated with neuronal differentiation by signalling through Shc/Grb2/m-Sos/Ras/Raf-1 [where Grb2 is the growth factor receptor-bound protein 2, Shc is an SH2 (Src homology 2)-containing adaptor protein that binds Grb2, m-Sos is a mammalian homologue of the *Drosophila* son of sevenless gene (a GDP-releasing factor of Ras) and Raf is the serine/threonine protein kinase family downstream of tyrosine kinases and upstream of MEK], PLC-γ1 (phospholipase C-γ1)/PKC (protein kinase C), Gab1 (Grb2-associated binder-1)/PI3K (phosphoinositide 3-kinase)/Akt (a product of the *v-akt* oncogene ≡ protein kinase B) and Crk/C3G/Rap1/B-Raf (where Crk is an oncogene, adaptor protein containing SH2 and SH3 domains and C3G is a guanine nucleotide-exchange factor that activates Rap1) [10]. Integration of the survival and differentiation responses occurs at MEK1/ERK1, leading to sustained ERK activation [9]. Several lines of evidence suggest that TrkA in endosomes activates Rap1 and B-Raf, which is responsible for sustained activation of MEK1/ERK1 [11–13]. Wu et al. [11] show that the TrkA in endosomes is a platform for a Crk/C3G/Rap1/B-Raf signalling complex, and Kao et al. [13] show that FRS2/SNT1 (an adaptor protein that

Abbreviations used: CIP, calf intestinal phosphatase; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; ERK1b, an alternatively spliced isoform of ERK1; Grb-2, growth factor receptor-bound protein 2; Ksr, kinase suppressor of Ras; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK kinase; MP-1, MEK partner 1; NGF, nerve growth factor; pERK, phosphorylated ERK; Raf, the serine/threonine protein kinase family downstream of tyrosine kinases and upstream of MEK; PI3K, phosphoinositide 3-kinase; TrkA, receptor tyrosine kinase for NGF.

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binds to Trk and other receptor tyrosine kinases) nucleates the complex. The Trk family of receptor tyrosine kinases appears to be unique in its ability to activate this pathway and cause sustained ERK activation. We hypothesize that MEK1/ERK1 multiprotein complexes participate in this unusual Trk-to-ERK pathway.

Additional spatial and temporal control of signalling cascades is achieved through alternative splicing, which gives rise to protein isoforms with structures that confer distinct binding properties. An example of this is ERK1b, an alternatively spliced isoform of ERK1 with a 26-amino-acid insertion [14]. ERK1b was previously referred to as ERK4 [15–17]. This isoform has a more restricted tissue distribution compared with ERK1; it is mainly found in heart, brain, lung and kidney, and is present in human and rat cell lines and human cancers [14,18]. ERK1b is activated by EGF (epidermal growth factor) and NGF in a similar way to ERK1, but its activity is sustained longer than ERK1 due to diminished interaction with deactivating phosphatase [17,19]. ERK1b also has a different intracellular distribution compared with ERK1, suggesting altered interaction with targeting proteins [14,19].

Discovery and characterization of scaffold proteins and their kinase binding partners has predominantly relied on tagged protein overexpression and immunoprecipitation or two-hybrid screens. The physical nature of endogenous scaffold-kinase complexes in living cells has been inferred to be something akin to a multisubunit enzyme, but the overall size and number of subunits are not known. Rat pheochromocytoma (PC12) cells have been used extensively to elucidate signalling events downstream of NGF's activation of TrkA, including ERK activation, as a model for signalling in neurons [20–22]. In the present study, we used cell fractionation and biochemistry without protein overexpression to characterize endogenous protein complexes from PC12 cells. Widely used kinase activity assays gave inconsistent results when analysing organelles in the high-speed pellet, which motivated a detailed analysis and modification of the techniques used to recover active kinases. This led to the discovery and characterization of two surprisingly large protein complexes, one containing ERK and MEK and another containing B-Raf.

EXPERIMENTAL

NGF treatment and *in vitro* reactions

PC12 cells, obtained from L. Greene (Columbia University, New York, NY, U.S.A.), were grown in RPMI 1640 medium, 10 % (v/v) horse serum, 5 % (v/v) fetal calf serum on collagen-coated tissue culture dishes as described in [23]. Cells were bound to 1 nM NGF (a gift from W. C. Mobley, Stanford University, Palo Alto, CA, U.S.A.) or 125 I-NGF at 4°C (prepared as described in [21]) and washed before initiating the intracellular response for 10 min at 37°C as described in [21]. Control or NGF-treated cells were resuspended in a cytoplasm-like buffer and subjected to a single passage through a Balch homogenizer, which tears the plasma membrane [24,25]. Mechanical permeabilization and *in vitro* reactions with an ATP-regenerating system were performed exactly as described in [22]. Cells were fractionated either directly after mechanical permeabilization (control) or after *in vitro* reactions for 15 min at 37°C with ATP.

Cell fractionation and velocity gradients

Fractionation of permeabilized semi-intact cells and of organelles that emerged from them was performed as described in [21,22]. PC12 cells incubated without or with NGF (1 nM) at 4°C were washed, warmed for 10 min (37°C), chilled (4°C) and mechanically permeabilized by a single passage through a Balch homo-

genizer. The semi-intact cells, containing the nucleus, plasma membrane, Golgi complex and other large or tethered organelles, were separated by centrifugation at 1000 *g* for 10 min and their membranes were extracted in PBS, 1 mM EDTA, 1 mM EGTA containing 1 % Nonidet P40 (referred to as Igepal; Sigma, St. Louis, MO, U.S.A.) and protease inhibitors, on ice for 1 h. The suspension was centrifuged at 10000 *g* for 10 min; the supernatant was defined as the P1M [detergent extract of the first pellet (P1)] fraction [21,22]. Organelles that emerged from permeabilized cells in the supernatant of the 1000 *g* spin (S1) were concentrated together to form P2' (a pellet formed by centrifugation at 100000 *g* without the intermediate 8000 *g* spin to form the second pellet P2) through a 0.4 ml pad of either 10 % sucrose, 20 mM Mops (pH 7.2), 1 mM EGTA, 1 mM sodium orthovanadate (Na_3VO_4) or 5 % (v/v) glycerol, 1 mM Na_3VO_4 in buffer B (a cytoplasm-like buffer containing 38 mM each of the potassium salts of aspartic, gluconic and glutamic acids, 20 mM Mops, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EGTA and 1 mM EDTA; pH 7.1). Where indicated, Igepal (1 %) was added to the S1, which was incubated for 1 h on ice before the 100000 *g* centrifugation to form the detergent-resistant P2'. Alternatively, large organelles were first separated by centrifuging at 8000 *g* for 35 min (forming P2) before the 100000 *g* centrifugation as above (forming P3 and the supernatant S3; [21,22]).

Sucrose gradients were performed as described in [21,22]. For iodixanol gradients, the 100000 *g* P2' (prepared over a sucrose pad) was resuspended in buffer B containing 5 mM GSH and applied to gradients of 0–30 % iodixanol (Optiprep™; Nycomed Pharma, Oslo, Norway) in buffer B and centrifuged at 200000 *g* for 1 or 3 h (defined as 200 and 600 *kg* · h respectively). For glycerol gradients, the 100000 *g* P3 (prepared over a glycerol pad) was resuspended with or without 0.4 % Igepal in buffer B containing 5 mM GSH. Samples were incubated on ice for 1 h, applied to gradients of 5–25 % glycerol in buffer B and centrifuged at 200000 *g* for 1 h [22]. Gradient fractions were collected from the bottom of the tube; proteins were precipitated by trichloroacetic acid, gamma radioactivity counted or subjected to SDS/PAGE and transferred on to nitrocellulose.

Western blots and kinase activity assays

Antibodies to ERK were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) or Cell Signaling Technology (Beverly, MA, U.S.A.). All three of them reacted with the slower migrating, alternatively spliced isoform of ERK1 (ERK1b). On NGF stimulation, ERK1b reacted with polyclonal anti-phospho-ERK but not with monoclonal (E10) anti-phospho-ERK (both from Cell Signaling Technology). Western blots were also probed with the following: anti-MEK1/2 (Cell Signaling Technology), anti-Raf-1, anti-B-Raf (Santa Cruz Biotechnology), anti-synaptophysin (SY38; Boehringer Mannheim), anti-TrkA (RTA; obtained from L. Reichardt, University of California, San Francisco, CA, U.S.A. [26] and Upstate Biotechnology), anti-MP-1 (Santa Cruz Biotechnology), anti-Ksr (Transduction Laboratories), anti-phosphotyrosine (4G10; obtained from M. Bishop, University of California), anti- α -tubulin (Sigma) and anti- γ -tubulin (Babco/Covance, Richmond, CA, U.S.A.). Antibodies were detected using horseradish peroxidase-coupled secondary antibodies and ECL® (Amersham Biosciences, Arlington Heights, IL, U.S.A.). Signal intensities from Western blots and in-gel kinase activity assays were measured from an X-ray film using NIH Image or from luminescence captured directly by a cooled charge-coupled-device camera (Fuji LAS-1000 or -3000) using Image Gauge software (Fuji Photo Film, Tokyo, Japan). ERK1 and ERK1b were

quantified together due to their close migration. Raw signals were corrected for the amount loaded on the gel and quantified as a percentage of the total in all fractions (P1M + P2 + P3 + S3). Quantitative comparison of immunoreactive proteins from several gradients is made possible by simultaneous probing of all blots with the same antibody solution and using the same exposure time.

In-gel kinase activity assays were performed based on a method described in [27]. MBP (myelin basic protein; 0.25 mg/ml) was added to the resolving SDS gel, the gel was run, then fixed in 20% (v/v) propan-2-ol and 50 mM Tris/HCl (pH 8.0), washed with 50 mM Tris/HCl (pH 8.0) and 5 mM 2-mercaptoethanol (buffer A), and then proteins therein were denatured in 6 M guanidinium chloride in buffer A for 1 h. Subsequent renaturation was achieved by soaking the gel in repeated changes of cold buffer A containing 0.04% Tween 40. The gel was washed with reaction buffer [40 mM Mops (pH 7.2), 15 mM MgCl₂, 1 mM MnCl₂ and 300 μ M Na₃VO₄], then incubated for 60 min at room temperature (22–25°C) in that buffer containing 50–75 μ Ci/10 ml [γ -³²P]ATP and 6–10 nM ATP (100:1 ratio of unlabelled to labelled ATP). The gel was washed extensively with 5% (w/v) trichloroacetic acid, 1% pyrophosphate, dried and exposed to an X-ray film. The specific activity of ERK1, defined as the ratio of kinase activity in a particular cell fraction using this assay divided by the amount of ERK1 in that fraction (as determined by Western blotting), was determined from three experiments where both ERK Western blots and in-gel kinase activity assays were performed in the same experiment.

The Elk1 phosphorylation assay was performed on 100 μ l of each cell fraction using the MAPK assay kit according to the manufacturer's instructions (Cell Signaling Technology). The reaction was terminated by the addition of SDS sample buffer and samples were Western blotted using anti-phospho-Elk1 (Cell Signaling Technology).

For phosphatase treatment, detergent-resistant P2' was incubated with CIP (calf intestinal phosphatase; New England Biolabs, Beverly, MA, U.S.A.). P2' was resuspended in CIP buffer [100 mM NaCl, 50 mM Tris (pH 7.9), 10 mM MgCl₂ and 1 mM dithiothreitol] and incubated at 37°C for 30 min with 20 units of CIP.

Modified immunoprecipitations

Immunoprecipitation with anti-TrkA (RTA) from cell fractions or detergent-soluble extracts from whole cells was performed using IP buffer [0.15 M NaCl, 1% Igepal, 0.5% deoxycholate, 20 mM Tris (pH 8) and 1 mM EDTA] containing 1% BSA. Vesicle pellets were resuspended directly in IP buffer; P1M and S3 fractions were diluted into it (1:10). After overnight antibody binding and 1–2 h incubation with Protein A–Sepharose (Pierce, Rockwood, IL, U.S.A.), IP buffer was used to wash antibody-bound Protein A beads. We were unable to co-immunoprecipitate ERK1 and TrkA from either cell fractions or detergent-soluble whole cell lysates under these conditions (results not shown). The discrepancy between our results and those showing that ERK co-precipitates with TrkA [28] is probably due to different antibodies and different detergents used for cell lysis, immunoprecipitation and washing the immunoprecipitate.

For salt disruption, 100 000 g P2' samples were resuspended in 5% glycerol and 1 mM Na₃VO₄ in buffer B, and NaCl was added as a solid and dissolved to a final concentration of 1 M. Samples were incubated on ice for 1 h, then diluted into lysis buffer as above, except that NaCl was not added so that the final salt concentration was 150 mM. Control samples were treated identically except that the salt was only present in the lysis buffer at 150 mM. Immunoprecipitation using immobilized phospho-

p44/42 ERK antibody was performed as above. Elk1 phosphorylation and in-gel kinase activity assays were performed on the samples as described above.

Anti-phospho-ERK immunoprecipitations were modified to promote recovery of kinase from the high-speed pellet. The 100 000 g P2' samples were resuspended in 50 mM Tris (pH 8.0) and 10 mM EDTA, and then lysis buffer containing 0.1% SDS was added. Control samples were resuspended in 50 mM Tris (pH 8.0) and 10 mM EDTA, and SDS was added to a concentration of 1%. The samples were boiled for 5 min, rapidly chilled on ice, and lysis buffer was added to dilute the SDS to a final concentration of 0.1%. Subsequent immunoprecipitations were performed as above.

For the anti-tubulin immunoprecipitations from gradient pools (Figure 6), a detergent-resistant P2' was prepared as above and resuspended in buffer B containing 5 mM GSH, protease inhibitors as above, phosphatase inhibitors (1 mM Na₃VO₄ and 0.1 μ M Calyculin A; Cell Signaling Technology) and taxol (20 μ M Paclitaxel; Cytoskeleton, Denver, CO, U.S.A.). Samples were applied to 0–30% iodixanol gradients in the same way as in Figure 5 but with a 0.25 ml 80% glycerol pad and centrifuged for 600 kg · h. Four fraction pools were collected from the bottom of the tube (fraction volumes 1.25, 1.5, 1.0 and 1.4 ml respectively). To fraction pools 1 and 4 (bottom and top of the gradients respectively), 50 mM NaCl, 1% BSA, 1% Igepal and 11% glycerol were added (for pool 1, glycerol was 20% of the total due to the gradient's pad). One-ninth of each pool was trichloroacetic acid-precipitated for gel electrophoresis. Pools were split and incubated with 20 μ g of anti-tubulin (mouse ascites DM1A; Sigma); controls received no antibody and showed no signal for B-Raf, MEK or ERK on Western blots (results not shown; Figure 6, MTIP, lanes E3 also serve as a control). Antibodies were collected by binding to Ultralink Immobilized Protein A/G beads (Pierce) and beads were washed with MTIP buffer [0.5 × buffer B; 50 mM NaCl, 11% glycerol, 16.7 mM Tris (pH 7.7), 0.33% Igepal and 0.3 mM EDTA], centrifuged through a 0.4 ml pad containing 20% glycerol, 50 mM NaCl and 0.5 × buffer B and resuspended in SDS/PAGE sample buffer for electrophoresis.

All experiments presented or discussed have been repeated two or more times with similar results.

RESULTS

ERK distribution and activity

To examine the role of ERK kinases in NGF signalling, we investigated their subcellular localization. We examined whether protein complexes containing ERK may be detected biochemically in cell fractionation experiments of PC12 cells treated with NGF. We hypothesized that endogenous protein complexes containing ERK kinases can be detected without overexpression of one or more components. Mechanical permeabilization was used as a very gentle homogenization that allows intracellular organelles and untethered cytoplasmic components to be recovered free of the plasma membrane [21]. A differential sedimentation method was used to separate cytoplasmic components that emerged from mechanically permeabilized cells (Figure 1). Previous work has demonstrated that the plasma membrane, nucleus and Golgi are found in the semi-intact cells, and their proteins are extracted into the P1M fraction; the low- and high-speed pellets (P2 and P3) contain large and small organelles that are released from permeabilized cells, respectively; P3 contains synaptic vesicles and transport vesicles [21,22,29].

The distribution of active ERK kinases in response to NGF treatment was determined by several methods (Figure 1). NGF caused

Figure 1 Distribution of ERK protein and activity after differential sedimentation

PC12 cells incubated without (CONTROL) or with NGF (NGF) were fractionated by differential sedimentation (see the Experimental section) [21]. The detergent-soluble membranes in the 1000 *g* pellet (P1M), the 8000 *g* pellet (P2), the 100 000 *g* pellet (P3) and the 100 000 *g* supernatant (S3) were subjected to SDS/PAGE, Western blotted with anti-ERK antibody (topmost panel) or anti-phospho-ERK (pERK, second panel) using ECL[®] for detection. Distribution of ERK kinase activity was assessed using an in-gel MBP kinase assay without immunoprecipitation [27] (third panel) and using immunoprecipitation with anti-phospho-p44/42 MAPK antibody, followed by phosphorylation with Elk1 substrate (bottom-most). The positions of molecular-mass standards (in kDa) for each panel set are indicated in the middle lane. Appropriate bands are identified as pERK1/2 (pERK1/2), active ERK (aERK1/2) and phosphorylated Elk1 (pElk1).

an increase in phospho-ERKs in all cell fractions, and induced 5–6% of the total ERK1 and ERK2 in the cells to move from the cytosol (Figure 1, lane S3) to the semi-intact cell extracts that contained nuclear proteins (P1M). In-gel kinase activity assays [27] indicated a similar distribution of ERK activity (Figure 1, third panel, lanes P1M and S3). Intriguingly, the specific activity of ERK proteins (the ratio of active ERK to total ERK) was highest in the high-speed pellet (Figure 1, P3), which contained 3–4% of ERK1 proteins and 5–7% of ERK2. A widely used ERK1 activity assay that uses immunoprecipitation with anti-phospho-ERK1 antibody and incubation with Elk1 as a substrate (Cell Signaling Technology) gave different results for the high-speed pellet. NGF-induced increases in ERK1 activity in the semi-intact cells extracts were noted (Figure 1, bottom-most, P1M),

but little activity was detected in the high-speed pellet (Figure 1, bottom-most, P3). Thus, whereas anti-phospho-ERK blots and the in-gel kinase activity assay indicated that highly active ERK kinases were present in the high-speed pellet, very little was immunoprecipitated.

Characterization of the ERK protein complex

We hypothesized that ERK in the high-speed pellet was present in a protein complex that prevented its immunoprecipitation. We attempted to dislodge ERK1 from these protein complexes using high-salt concentrations, which are supposed to disrupt protein–protein interactions. Salt treatment improved recovery of ERK1 significantly (Figure 2A). Interestingly, MEK1 co-precipitated with phospho-ERK1 with or without salt treatment; more MEK1 was recovered in the salt-treated samples (Figure 2A). The results suggest that ERK and MEK were released from a protein complex by high salt to allow antibody binding and then re-associated during the incubation after the salt concentration was lowered. ERK kinases remained in the high-speed pellet after treatment with non-ionic detergent, which is also consistent with their being in a protein complex (Figure 2E).

ERK activity was recovered from high-speed pellet samples immunoprecipitated after salt treatment (Figure 2B). Activity was detected using the in-gel kinase activity assay, probably because proteins were allowed to renature as part of the assay (Figure 2B, upper panel); salt treatment caused a decrease in activity by the Elk1 phosphorylation assay, which may be due to partial denaturation (Figure 2B, lower panel). We explored other methods to dislodge active ERK from the high-speed pellet and discovered that the addition of SDS to the immunoprecipitation buffer allowed recovery of NGF-activated ERK kinases as shown by the Elk1 phosphorylation assay (Figure 2C).

In addition to ERK1 and ERK2, we noted an isoform of ERK1 that migrated slightly more slowly on SDS gels compared with purified phospho-ERK1 (Figure 2D, ERK1b). Phosphatase treatment removed phosphates from ERK1, ERK2 and MEK1 [Figure 2E, pERK (phosphorylated ERK) and pMEK panels, + CIP], but did not affect their electrophoretic mobility or decrease the amounts of this slower migrating isoform (Figure 2E, topmost panel, + CIP, ERK1b). Evidence from several experiments suggests that this slower migrating band is the alternatively spliced isoform of ERK1, ERK1b, which is predicted to have a molecular mass of 45.6 kDa [14,19]. This isoform was enriched in the 100 000 *g* pellet (P2' or P3), and Western blotted with several different antibodies to ERK1 (see the Experimental section). ERK1b immunoprecipitated with (Figure 2A), but did not blot well with, monoclonal anti-phospho-ERK (Figure 2E). ERK1b reacted with anti-phosphotyrosine (Figure 2E, pTyr) and weakly with polyclonal anti-phospho-ERK (see below). These results demonstrate that the slower migrating isoform is not hyper-phosphorylated and are entirely consistent with all previous results for ERK1b/ERK4' in extracts from stimulated PC12 cells [14,17]. Loss of gel resolution occurred during the denaturation and renaturation procedure in the in-gel kinase activity assay, so we could not distinguish the relative activity of the two closely migrating ERK1/ERK1b bands (Figure 2B).

Previous work demonstrates that brief incubations of permeabilized cells with an ATP-regenerating system act as an *in vitro* chase to events initiated *in vivo* [22,29]. In the present study, we used *in vitro* reactions to induce dynamic changes in ERK distribution and activity. We found that *in vitro* reactions did two things to the ERK kinases. First, the amounts of phosphorylated ERK1b, ERK1, ERK2 and MEK1 were diminished by *in vitro* reactions (Figure 2E, pTyr, pERK, pMEK and + ATP). This was

Figure 2 Salt and detergent treatment of high-speed pellet

(A) Salt disruption improves immunoprecipitation. High-speed pellets (P2') which contain all components in P2 and P3 fractions in Figure 1 [21] were incubated without (– NaCl) or with 1 M NaCl (+ NaCl) and immunoprecipitated (after dilution of salt) with anti-phospho-ERK. Samples were Western blotted with anti-ERK (upper panel) and anti-MEK (lower panel). Cell extracts from control cells without immunoprecipitation (P1M, no IP) are shown for comparison. Control or NGF-treated cells (– or + NGF respectively) were permeabilized and, where indicated (+ ATP), subjected to 15 min *in vitro* reactions with an ATP-regenerating system. ERK1, ERK2 and the slower migrating isoform of ERK1 (ERK1b) are indicated, in addition to MEK1 and the protein p35, which also blots with anti-phospho-MEK (shown in E). (B) The kinase activity of NGF-treated immunoprecipitated samples in (A) was measured as in Figure 1 by the *in-gel* MBP kinase assay (upper panel) and Elk1 substrate phosphorylation (lower panel). (C) Mixed micelle immunoprecipitation. P2' pellets were resuspended in Tris buffer, then diluted into immunoprecipitation buffer containing 0.1% SDS in a mixed micelle with other detergents (–) or boiled in a buffer containing 1% SDS, after which the SDS concentration was diluted to 0.1% in immunoprecipitation buffer (+ boiled). Samples were immunoprecipitated with anti-phospho-ERK and an Elk1 phosphorylation assay was performed. Kinase activity was eliminated by boiling in SDS. (D) P2' pellet immunoprecipitated with anti-phospho-ERK after SDS treatment as in (C) when compared with the same gel with purified phospho-ERK1 (Cell Signaling Technology), blotted with anti-ERK. (E) Phosphatase treatment does not alter the mobility or amount of ERK isoforms. S1 prepared from NGF-treated cells without (–) or with *in vitro* reactions (+ ATP) was incubated with non-ionic detergent before centrifugation to form the DR (detergent-resistant) pellet (DR P2') and supernatant (S2'). DR P2' fractions were analysed directly or subjected to incubations with CIP (+ CIP) before gel electrophoresis. SDS/PAGE was performed on 1/80 of the total S2' fraction for comparison with 1/2 of DR P2'. Western blotting was performed with anti-ERK, anti-phospho-ERK (pERK), phosphotyrosine (using the monoclonal 4G10; pTyr) phospho-MEK (pMEK) or α -tubulin.

apparently due to the action of cellular phosphatases, since the amount of ERK1 and ERK2 did not change (Figures 2A and 2E, topmost panel). Interestingly, the NGF-stimulated ERK activity in the high-speed pellet remained after *in vitro* reactions (Figure 2C). Secondly, *in vitro* reactions caused amounts of ERK1b to increase in the high-speed pellet (Figures 2A and 2E, topmost panel).

We characterized further the protein complex containing ERK and MEK by subjecting the high-speed pellet to velocity sedimentation through glycerol gradients (Figure 3). Some ERK1 and ERK2 remained at the top of glycerol gradients (fractions 21–25), but almost all of the ERK1b migrated to the bottom of the gradients, with a minor peak at fractions 7–8 (Figure 3A). The amount of ERK1 and ERK1b that migrated to the bottom of the gradients increased in response to NGF (Figure 3A). The mobility of ERK1 and ERK1b on glycerol velocity gradients was not affected when the high-speed pellet was treated with non-ionic detergent (Figure 3B), indicating that they are not attached to a membrane-bound organelle. In contrast, synaptic vesicles and larger vesicles containing synaptophysin (p38) were completely solubilized by the detergent, which is indicated by the absence of synaptophysin in all the fractions except those at the top of the gradient after detergent treatment (fractions 21–25; Figure 3C). MEK1 was also present in the detergent-resistant protein complex that migrates to the bottom of glycerol gradients (Figure 3B). These results explain the poor immunoprecipitation of ERK1 in the high-speed pellet in the absence of salt treatment (Figures 1 and 2A); ERK-associated proteins do not dissociate from their protein complex in the presence of non-ionic detergent, and other bound proteins probably impede antibody access.

ERK-containing particles and microtubules

A fraction of ERK proteins has been shown to be bound to microtubules in PC12 cells [16] as well as in neurons and other cell types [30,31], so it is possible that ERKs are bound to microtubule fragments in the high-speed pellet. Microtubules mostly depolymerized during the mechanical permeabilization procedure at 4°C; most of the α -tubulin was found in the cytosol (Figure 2E, bottom-most, S2'), with some remaining in the semi-intact cells (results not shown). Tubulin was detected in the high-speed pellet and *in vitro* reactions increased the amounts by 2–5-fold in this fraction (Figure 2E, bottom-most), suggesting that microtubule fragments partially assembled during these reactions and migrated into the high-speed pellet. Considering that ATP can be converted into GTP and that GTP is present in cell extracts, these results are consistent with nucleotide requirements for microtubule polymerization *in vitro* [32].

The distribution of microtubules in glycerol gradients was somewhat similar to that of ERK (α -tubulin, Figure 3D). Signalling events activated by NGF enhanced microtubule polymerization or stability, indicated by increased amounts of microtubule fragments at the bottom of the glycerol gradient (fractions 1–4, Figure 3D, lower panel). The γ -tubulin ring complex is a cold-stable microtubule-nucleating particle of approx. 36 S [33]. This complex, visualized by probing Western blots of glycerol gradient fractions with anti- γ -tubulin, migrated slightly less rapidly (fractions 13–15, Figure 3E) compared with synaptic vesicles (fractions 10–13, Figure 3C), which are approx. 50 S [34]. γ -Tubulin was also detected in ERK-containing fractions at the bottom of the gradient, and amounts in these fractions increased after *in vitro* reactions (Figure 3E). Since ERK was not in the same fractions (Figure 3A), the results suggest that ERK is not bound to the γ -tubulin ring complex itself. ERK proteins cosedimented at the bottom of glycerol gradients (fractions 1–4, Figure 3) with fragments of microtubules containing both γ - and

Figure 3 Glycerol velocity gradients of the high-speed pellet

(A) Cells were treated with NGF (NGF) or vehicle (control) and subjected to *in vitro* reactions (+ATP) and differential sedimentation. High-speed pellet (P3) samples were applied to glycerol gradients [22]. Gradient fractions were Western blotted with anti-ERK. Fraction numbers are labelled above each lane. Cell extracts (P1M) are shown on ERK blots to indicate the positions of ERK1b, ERK1 and ERK2. (B) Detergent solubilization did not affect the sedimentation of ERK1, ERK1b or MEK1 to the bottom of glycerol velocity gradients. Where indicated (+detergent), P3 samples as in (A) (+NGF) were incubated with non-ionic detergent before glycerol gradient sedimentation. Blots of gradient fractions were probed with anti-ERK1 (upper panel) and anti-MEK (lower panel). (C) Synaptic vesicles and other vesicles containing synaptophysin were

Figure 4 ERK and tubulin localization on iodixanol velocity gradients

Cells were treated with NGF and fractionated before (NGF) or after (NGF + ATP) *in vitro* reactions. The high-speed pellet (P2') was prepared as in Figure 2(A) and applied to 200 kg · h iodixanol gradients. Gradient fractions were collected from the bottom, so bodies of larger mass are in lower-numbered fractions. Gradient fractions were Western blotted with anti-ERK (A) and anti- α -tubulin (B). ERK-containing peaks are labelled E1–E3.

α -tubulin that are probably protofilaments nucleated by the γ -tubulin ring complex [35]. It is possible that ERK and MEK in the high-speed pellet are bound to these microtubule fragments.

Glycerol gradients do not resolve larger microtubule fragments well, so iodixanol velocity gradients were used to examine ERKs and microtubules in the high-speed pellet (Figure 4). These gradients resolved three peaks containing ERK kinases from the NGF-treated P2' fraction (labelled E1–E3, Figure 4). Without *in vitro* reactions, peak E3 contained the greatest quantities of ERK1b, ERK1 and ERK2 (Figure 4A, upper panel). Microtubules of discrete lengths (i.e. microtubule fragments at intermediate states of polymerization) were also resolved, and three of these peaks exactly co-migrated with ERKs (peaks E1, E2 and E3; Figure 4B, upper panel). There was an additional tubulin peak that did not contain ERKs (fractions 20–22, Figure 4B, lower panel).

If ERK proteins shift in sedimentation velocity along with tubulin after microtubule polymerization, then this is strong evidence that they are bound to microtubules. Conversely, if ERKs do not shift into microtubule-containing fractions, this indicates that they are not bound. Microtubule polymerization was stimulated by *in vitro* reactions, indicated by depletion of short fragments at the top of the gradient (peak E3; Figure 4B, lower panel) and enrichment in longer fragments (peak E1). Dynamic changes in the migration of ERK1b closely paralleled those of microtubule fragments (Figure 4A, +ATP). The identical shift of ERK1b from peak E3 to peak E1 after *in vitro* reactions is strong evidence

solubilized by detergent treatment. P3 samples (+NGF + ATP) were untreated or treated with detergent as in (B), and blots of gradient fractions were probed with anti-synaptophysin (p38). Synaptic vesicles (fractions 10–13) are indicated. (D) Distribution of microtubule fragments on glycerol gradients. Glycerol gradient samples as in (A) were probed with anti- α -tubulin. (E) P3 samples from NGF-treated cells without (NGF) or with (NGF + ATP) *in vitro* reactions were resolved on glycerol velocity gradients and Western blots were probed with anti- γ -tubulin. The position of the 36 S γ -tubulin ring complex is noted (γ -tub RC, fractions 13–15).

that ERK1b is bound to microtubule fragments that are partially polymerized during the reactions.

ERK1 and ERK2, however, do not seem to be predominantly bound to microtubules, since only a small amount of ERK1 and ERK2 shifted to peaks E1 and E2 on microtubule polymerization (Figure 4A, lower panel). Most of ERK1 and ERK2 remained in peak E3 after *in vitro* reactions, whereas the vast majority of tubulin was depleted from these fractions (Figure 4B, lower panel). We therefore conclude that ERK1 and MEK1 are present in a large protein complex that is independent of microtubules, which we will call a particle. The particles were found in the 100 000 *g* pellet, were resistant to non-ionic detergent, sensitive to high salt and migrated to the bottom of glycerol velocity gradients and near the top of iodixanol velocity gradients.

The size of the particles suggests the hypothesis that they may contain other signalling and scaffolding components. As noted above, MEK1 and ERK2 were also present in microtubule-independent particles that are the same size; their universal comigration under different conditions and co-immunoprecipitation (Figure 2) suggest that they are part of the same complex. The particles did not contain MAP2, which was associated with polymerized microtubules predominantly in peak E1, nor did they contain p70 ribosomal S6 kinase (results not shown). Neither of the scaffolding proteins known to bind both ERK and MEK, Ksr or MP-1, was detected in either the microtubule-containing fractions or the microtubule-independent particles, although they were detected in other fractions (P1M, S1 and S3; M. L. Grimes, unpublished work). The results suggest that the particles described here are distinct from previously described scaffolded kinase assemblies. Thus it is possible that they also contain upstream kinases.

B-Raf is in different particles

B-Raf has been implicated in NGF's activation of a sustained ERK response initiated from endosomal receptors [11–13], so we asked whether B-Raf is associated with ERK/MEK particles. B-Raf was found in detergent-resistant cytoplasmic particles whose distribution on iodixanol gradients was similar to that of ERK and MEK (fractions 20–23, Figure 5A). Some B-Raf was also detected in a distinct peak in the middle of the gradients (fractions 15–19). The B-Raf and MEK1/ERK1 peaks were reproducibly offset from one another by one fraction on these gradients, which could indicate poor resolution of two distinct kinds of particles. If the force was tripled, the B-Raf and MEK1/ERK1 particles were pushed further into the gradients (Figures 5C–5F). The second B-Raf peak migrated to fractions 10–12 under these conditions (Figures 5C–5F). Although the smallest B-Raf particles (fractions 16–20) were physically similar in many respects (detergent resistance and sedimentation characteristics), the 600 kg · h iodixanol gradients show that they are slightly larger than, and thus distinct from, ERK1/MEK1 particles (fractions 19–22). The profiles of ERK1 and MEK1 matched one another exactly under all conditions (Figures 5C–5F). In addition, NGF caused the assembly of particles containing ERK1 and MEK1 (peak E3) and decreased the amounts of both B-Raf-containing peaks (Figures 5C and 5D). Thus B-Raf and ERK/MEK are present in two distinct detergent-resistant particles whose assembly appears to be regulated in opposite ways by NGF.

Microtubule fragments were pushed to the bottom of 600 kg · h gradients (Figure 5B, fractions 1–5). *In vitro* reactions increased the amount of microtubule fragments, and NGF amplified microtubule polymerization (Figure 5B). Some B-Raf, MEK and ERK co-localized with microtubules at the bottom of the gradients (Figures 5C–5F, fractions 1–5, peak E1).

Figure 5 Separation of detergent-resistant particles containing ERK, MEK and B-Raf on iodixanol velocity gradients

The detergent-resistant high-speed pellet (DR P2') as in Figure 2E) from PC12 cells with or without NGF treatment or *in vitro* reactions was analysed on 200 kg · h (A) or 600 kg · h iodixanol gradients (B–F). Chemiluminescent signals for B-Raf, MEK, ERK1 and α -tubulin from gradient Western blots were quantified using a cooled charge-coupled-device camera (Fuji LAS-1000) and image analysis software (Fuji Image Gauge). Absorbance units with background subtracted ($\times 10^4$ for B-Raf; 10^3 for ERK1, MEK1 and tubulin) are plotted against fraction number. (A) Apparent co-localization of B-Raf with ERK1/MEK1 particles on 200 kg · h iodixanol velocity gradients in particles from NGF-treated cells as in Figure 4(A, upper panel). Gradient fractions were Western blotted with anti-B-Raf, and -MEK1. B-Raf ($\blacklozenge$) overlapped with MEK1 ($\square$) and ERK1 (results not shown for clarity) in the lightest peak (fractions 21–24). B-Raf was detected in an additional peak that sedimented more rapidly (fractions 15–19). (B) Microtubule fragments migrated to the bottom of gradients centrifuged at 200 000 *g* for 3 h (600 kg · h). Tubulin was quantified from Western blots of gradient fractions on detergent-treated P2' samples derived from untreated cells without ($\blacktriangledown$) or with ($\blacklozenge$) *in vitro* reactions, and NGF-treated cells without ($\square$) or with ($\triangle$) *in vitro* reactions. Note that the greatest amount of microtubule fragments in fractions 1–5 was detected after NGF treatment and *in vitro* reactions ($\triangle$). (C–F) Under all conditions, microtubule-independent particles containing ERK1 and MEK1 had exactly the same distribution, whereas B-Raf particles were slightly larger. B-Raf ($\blacklozenge$), MEK1 ($\square$) and ERK1b/ERK1 ($\triangle$, quantified together) were quantified from 600 kg · h gradients (as in B) from untreated cells without (C) or with (E) *in vitro* reactions, and NGF-treated cells without (D) or with (F) *in vitro* reactions. Peaks E1 and E3 were pooled as shown for the microtubule immunoprecipitations in Figure 6.

Microtubule-bound and microtubule-independent particulate kinases

We sought to determine directly which kinases were bound to microtubules, which kinases were in microtubule-independent particles and where the phosphorylated (i.e. activated) ERKs are found. We used the same conditions as in Figure 5, with and without NGF treatment and *in vitro* reactions, and separated ERK/MEK and B-Raf particles from microtubules in the detergent-resistant high-speed pellet using 600 kg · h iodixanol gradients. Anti-tubulin immunoprecipitations were performed from pools corresponding to fractions 1–6 (lowest, E1) and 17–23 (top-most, E3; Figure 6). *In vitro* reactions induced microtubule polymerization and increased the recovery of microtubule-bound proteins at the bottom of the gradients (Figure 6, + ATP, lanes E1). ERK1b was preferentially bound to microtubules, and it migrated to the bottom of these gradients and co-immunoprecipitated with microtubule fragments (Figure 6, second panel, lanes E1). Some,

Figure 6 Co-precipitation of kinases with microtubules

The detergent-resistant P2' from untreated or NGF-treated cells before (no reaction) or after *in vitro* reactions (+ATP) was fractionated on 600 kg·h iodixanol gradients as in Figure 5. Pools corresponding to fractions 1–6 (bottom, E1) and 17–23 (top, E3) were collected as shown in Figure 5. (Note that peak E2 merges with E1 on 600 kg·h gradients.) One-ninth of each sample was trichloroacetic acid-precipitated (input) and, on the remainder, immunoprecipitations with anti-tubulin were performed (MTIP). Western blots were probed with polyclonal anti-phospho-ERK, -ERK, -MEK and B-Raf (indicated). As shown in Figure 3, ERK1 and ERK2, but not ERK1b, were dephosphorylated by cellular phosphatases during *in vitro* reactions (topmost panel, +ATP). MEK2 appeared to bind preferentially to microtubules, as did ERK1b (third panel, lanes E1).

but not all, of the B-Raf, MEK1, ERK1, and a small amount of ERK2, were also found at the bottom of the gradients bound to microtubule fragments (Figure 6, lanes E1).

No kinases from fractions at the top of gradients immunoprecipitated with anti-tubulin, confirming that B-Raf, MEK1, ERK1 and ERK2 were in microtubule-independent particles (Figure 6, lanes E3). ERK1b was not present in microtubule-independent particles. As shown also in Figure 5, NGF caused an approx. 2-fold increase in the amount of ERK1 and MEK1 in microtubule-independent particles and B-Raf decreased in these particles after NGF treatment (Figure 6, lanes E3).

NGF activated ERK1 and ERK2 in the particles (Figure 6, topmost panel). The ratio of phosphorylated ERK1 and ERK2 (topmost panel) to total ERK (second panel) was by far the highest in microtubule-independent particles after NGF treatment (Figure 6, no reaction, NGF, input, E3). Smaller amounts of phospho-ERK1b, -ERK1 and -ERK2 were found in the microtubule-bound fraction (Figure 6, topmost panel, no reaction, NGF, lanes E1). These results establish that ERK kinases activated by NGF are present in signalling particles that are independent of microtubules.

DISCUSSION

The present study has identified a previously uncharacterized cytoplasmic particle containing ERK and MEK. The particles are distinct from fragments of microtubules and distinct from slightly larger particles containing B-Raf. NGF caused both the amount and the kinase activity of ERK1 to increase in the particles, indicating that they are an important intermediate in signal transduction. Signalling particles were resistant to non-ionic detergents and sensitive to high salt, which suggests that their assembly is governed by protein–protein interactions. The signalling particle described here appears to be a large, com-

plex 'protein machine' that probably has an elaborate list of components and intricate regulation [36], similar in concept to a 'signalosome' or 'transducisome' [37,38], although it apparently lacks membranes and as such does not fit classification as an organelle.

ERK-containing signalling particles are surprisingly large, much larger than expected for a ternary complex containing, for example, ERK1, MEK1 and MP-1 [5] or even a dimer of these [39]. The microtubule-independent particles were more rapidly sedimenting compared with 50 S synaptic vesicles and the 36 S γ -tubulin ring complex on glycerol velocity gradients (Figure 3), and had very similar velocity sedimentation characteristics to NGF- and TrkA-containing signalling vesicles on glycerol gradients and sucrose gradients [22]. ERK/MEK particles were less rapidly sedimenting compared with transport vesicles on 600 kg·h iodixanol gradients (M. L. Grimes, unpublished work). The results indicate that the size of ERK/MEK particles is 60–75 S.

We expected to find scaffolding proteins that bind ERK and MEK in the signalling particles, but we could not detect either Ksr or MP-1 despite many attempts. Both of these scaffolding proteins could be detected in detergent-solubilized cell extracts and in the soluble pool (P1M, S1 or S3 fractions respectively), but not in the detergent-resistant particles (results not shown). Recent work suggests that MP-1 mediates EGFR (EGF receptor) signalling from late endosomes. Wunderlich et al. [7] found a novel protein, p14, which binds to MP-1 and is localized in late endosomes and endosomal carrier vesicles. The MP-1–p14 complex is required for the localization of activated ERK to late endosomes and for a longer period of ERK signalling [6]. Ksr, on the other hand, associates with Raf-1 and MEK (and other proteins) in the cytoplasm and is recruited to the plasma membrane in response to Ras activation and protein phosphatase 2A's dephosphorylation of the site phosphorylated by the regulatory kinase C-TAK1 (Cdc25C associated protein kinase-1) [1,8]. Raf-1 was predominantly located in the P1M and cytosol fractions and was only weakly detected in detergent-sensitive endocytic organelles (results not shown). These results suggest that Raf-1 is membrane-bound and not in ERK particles. That the signalling particles described here appear to be distinct from scaffolded complexes containing Ksr and MP-1 and assemble in response to NGF suggests the hypothesis that the particles participate in sustained ERK activation, which response is unique to the Trk family of receptor tyrosine kinases [9].

B-Raf, an important intermediary for sustained ERK activation, was also found in detergent-resistant, microtubule-independent particles whose sedimentation velocity was greater than ERK/MEK particles. B-Raf has been shown previously to be in a large protein complex containing Hsp90 (heat-shock protein 90) and 14-3-3 [40]. Mutational disruption of the complex between B-Raf and 14-3-3 causes dysfunctional NGF signalling in PC12 cells [41], suggesting that this complex is important for effective signalling. B-Raf has been shown to interact with MEK1 under two-hybrid conditions [42], and indeed must interact at some point to carry the signal further down the kinase cascade, but it is not clear whether or how particles containing B-Raf interact with the distinct particles containing ERK and MEK. It may be that B-Raf is released from its complex when activated and, subsequently, transiently interacts with ERK particles. This possibility is suggested by the NGF-induced decrease in the amount of B-Raf in its microtubule-independent particles (Figures 5 and 6), and the small NGF-induced shoulder in the B-Raf peak that overlaps with ERK particles on iodixanol gradients (Figure 5D).

Microtubules could conceivably act as a scaffold to promote kinase–kinase interactions [43]. The interaction of ERK proteins with microtubules has been well documented. Co-staining of ERK

with microtubules has been observed in neurons and cultured cells by immuno-fluorescence and -electron microscopy [16,30,31]. Morishima-Kawashima and Kosik [16] showed that ERK1 and ERK1b/ERK4' were tightly bound to rat brain microtubules and resisted extraction with 0.35 M NaCl. We found that some, but not all, detergent-resistant ERK1, ERK2, MEK1 and B-Raf in the high-speed pellet were bound to or co-sedimented with microtubule fragments. It is possible that these kinases will link NGF signalling to microtubule polymerization, stability or movement [44]. In any case, our results show that microtubules are not the sole source of scaffolded kinases, since we could separate signalling particles from microtubule fragments by their distinct sedimentation velocity on iodixanol gradients (Figures 4–6).

The distinct sedimentation of ERK1b under conditions that fractionate signalling particles away from microtubules indicates that ERK1b is not in the same signalling particle as ERK1 and MEK and prefers to bind microtubules. ERK1b was tightly bound to a protein complex under all conditions; very little was free in the cytosol or immunoprecipitable without salt disruption (Figure 2A). *In vitro* reactions apparently cause ERK1b to be recruited into the high-speed pellet from the cytosol, as indicated by the loss of ERK1b from the S2' and increase in the P2' fraction (Figures 2A and 2E, topmost panel). Yung et al. [19] show that ERK1b binds less well than ERK1 to MEK in non-stimulated cells, which results in more prominent nuclear localization and also a diminished capacity to bind to the phosphatase PTP-SL [STEP (striatal-enriched phosphatase)-like PTP (protein tyrosine phosphatase)], which results in a more prolonged activation after EGF stimulation. ERK1b's resistance to endogenous or added phosphatases (Figures 2C, 2E and 6, and [19]), its relative enrichment in salt-washed microtubule pellets [16] and its preferential association with microtubules (Figures 5 and 6) suggest that it may be the major constitutively active MAPK that associates with microtubules [16]. Taken together, these results suggest that ERK1b has a unique set of binding interactions that govern its spatial and temporal responses to stimulation.

The signalling particles described here may be a means of sequestering or transporting ERK activity [43]. The topological problems of regulating and transporting ERK kinases are more profound in neurons than in cells without long processes. ERKs play a role in integrating multiple signal inputs in neurons to dictate responses as diverse as reversal of apoptosis and enhancement of long-term potentiation [20]. It is possible that microtubules play a role in the retrograde transport of downstream kinases; Johanson et al. [45] have detected, distal to a nerve crush of rat sciatic nerve, a build-up of PI3K, MEK kinase, MEK1 and ERK1, and Averill et al. [46] have detected retrograde transport of active ERK1 in dorsal root ganglion cells. It has been suggested that ERK fractionation into the high-speed pellet could be due to association with retrogradely transported TrkA-containing signalling vesicles [21,22,28,47]. We have shown here that the ERK/MEK particles are not organelles, however, and we could not detect ERK in TrkA immunoprecipitates or TrkA in ERK immunoprecipitates in the high-speed pellet or any other cell fraction (results not shown; see the Experimental section). Kinases, either individually or linked together by scaffold proteins in a signalling particle, may be conveyed on microtubules to different cellular locations to perform particular signalling tasks.

In addition to moving kinases around inside cells, signalling particles probably regulate activity in other ways. Previous studies suggest a role for protein complexes in the regulation of ERK activity and nuclear localization; pERK2 forms dimers, which are imported into the nucleus [39], and interaction of ERK with MEK enforces cytoplasmic as opposed to nuclear localization

[48]. Similar protein complexes may be present in other cell types; particles with similar characteristics to those described here and containing MEK and ERK were detected without overexpression of any signalling component in human neuroblastoma (SY5Y) and mouse pituitary (AtT-20) cells (M. L. Grimes, unpublished work); and Boulton and Cobb [49] demonstrated ERK1 and ERK2 in a particulate fraction of Rat 1 HIRc B cells, and the amounts increased transiently after insulin treatment. In addition, our results could explain why ERK activity failed to immunoprecipitate from high-molecular-mass complexes in previous studies [49,50].

Our studies suggest that signalling particles are large, complex protein machines, which contain protein kinases and as-yet-unidentified scaffold and other components that probably influence a signal-transduction pathway's fidelity, fecundity, location and longevity [1]. Many questions remain about the components, assembly, regulation and function of the ERK/MEK- and B-Raf-containing particles and the interactions between the two types of particles. Two technical considerations arise. First, immunoprecipitation of proteins from such a large, tightly bound protein complex can certainly be problematic. Secondly, physical characterization of such particles from cells overexpressing tagged proteins must be interpreted with caution, due to unknown effects from the interference of the tag with protein–protein interactions, or artifacts from unbalanced expression levels. Here, the particles were characterized at endogenous expression levels, albeit in tumour cells. Future studies on purified particles will help to elucidate other components and facilitate discovery of their regulatory mechanisms.

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