

# Intracellular Aggregation of Polypeptides with Expanded Polyglutamine Domain Is Stimulated by Stress-activated Kinase MEKK1

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**Abstract.** Abnormal proteins, which escape chaperone-mediated refolding or proteasome-dependent degradation, aggregate and form inclusion bodies (IBs). In several neurodegenerative diseases, such IBs can be formed by proteins with expanded polyglutamine (polyQ) domains (e.g., huntingtin). This work studies the regulation of intracellular IB formation using an NH<sub>2</sub>-terminal fragment of huntingtin with expanded polyQ domain. We demonstrate that the active form of MEKK1, a protein kinase that regulates several stress-activated signaling cascades, stimulates formation of the IBs. This function of MEKK1 requires kinase activity, as the kinase-dead mutant of MEKK1 cannot stimulate this process. Exposure of cells to UV irradiation or cisplatin, both of which activate MEKK1, also augmented the formation of IBs. The polyQ-containing huntingtin fragment exists

in cells in two distinct forms: (a) in a discrete soluble complex, and (b) in association with insoluble fraction. MEKK1 strongly stimulated recruitment of polyQ polypeptides into the particulate fraction. Notably, a large portion of the active form of MEKK1 was associated with the insoluble fraction, concentrating in discrete sites, and polyQ-containing IBs always colocalized with them. We suggest that MEKK1 is involved in a process of IB nucleation. MEKK1 also stimulated formation of IBs with two abnormal polypeptides lacking the polyQ domain, indicating that this kinase has a general effect on protein aggregation.

**Key words:** protein aggregation • inclusion body • polyglutamine • MEKK1 • stress

## Introduction

Abnormal polypeptides often emerge in mammalian cells as a result of denaturation due to stressful environments, chemical modifications, or mutations. Normally, cells can cope with accumulation of damaged proteins by either refolding them in chaperone-dependent processes or degrading them via the ubiquitin–proteasome system. However, if the cell's ability to refold or degrade abnormal polypeptides is exceeded, the denatured or partially unfolded molecules tend to aggregate (Dubois et al., 1991; Gragerov et al., 1991; Cotner and Pious, 1995; Kampinga et al., 1995; Johnston et al., 1998; Wigley et al., 1999) and form large inclusion bodies (IBs)<sup>1</sup> (Prouty et al., 1975; Klemes et al., 1981; Gragerov et al., 1991; Rinas and Bailey, 1993). It was

initially assumed that these IBs result simply from the intrinsic ability of denatured proteins to associate with each other until the aggregates become insoluble. However, recent findings indicate that IB formation in mammalian cells is a complex process that may actively involve cellular components. In the cytoplasm, proteins form small aggregates that are then transported along the microtubules into the centrosome (Johnston et al., 1998; Anton et al., 1999; Garcia-Mata et al., 1999; Vidair et al., 1999; Wigley et al., 1999; Fabunmi et al., 2000). Resulting IBs also incorporate heat shock proteins, ubiquitin, ubiquitin-conjugating enzymes, and 26S proteasomes (Wojcik et al., 1996; Anton et al., 1999; Garcia-Mata et al., 1999; Vidair et al., 1999; Wigley et al., 1999; Fabunmi et al., 2000), forming a large structure recently termed the “aggresome” (Johnston et al., 1998).

In a wide variety of hereditary diseases, abnormal polypeptides, which play a critical role in pathogenesis, accumulate in cells in a form of insoluble IBs. Neurological diseases associated with appearance of IBs in neurons include Alzheimer's disease, Parkinson's disease, and Huntington's disease, among others. IBs formed by different abnormal proteins in the course of such diseases have

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<sup>1</sup>Abbreviations used in this paper: CFTR, mutant ( $\Delta F508$ ) cystic fibrosis transmembrane conductance regulator; ERK, extracellular signal-regulated kinase; GFP, green fluorescence protein; HEK, human embryonic kidney; Hsp, heat shock protein; IB, inclusion body; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, MAPK kinase kinase; polyQ, polyglutamine.

many common features, including recruitment of components of the ubiquitin–proteasome-dependent degradation pathway and molecular chaperones, which suggests that such IBs may be similar to the aggresome.

Several diseases, including Huntington's disease (Bao et al., 1996; Gusella and Macdonald, 1998) and spinocerebellar ataxias (Cummings et al., 1999a), are associated with accumulation of abnormal proteins containing a polyglutamine (polyQ) domain. Expansion of such polyQ domain beyond the normal length results in abnormal protein conformation, which leads to aggregation as well as sequestration of various cell proteins (Bates et al., 1998). In the case of huntingtin, its proteolytic cleavage is an important factor promoting aggregation in neurons (Goldberg et al., 1996; Difiglia et al., 1997; Kahlem et al., 1998a; Wellington et al., 1998). Smaller polyQ-containing fragments of 350-kD huntingtin are more prone to aggregate, they easily form  $\beta$  sheets (Perutz, 1999), and may be cross-linked by transglutaminase (Kahlem et al., 1996, 1998a,b). The formation of IBs generally precedes neuronal death, which has led to the suggestion that formation of the aggregates may trigger neuropathology. On the other hand, there is much evidence that aggregation of polyQ-containing polypeptides does not contribute to pathogenesis (Klement et al., 1998; Saudou et al., 1998; Cummings et al., 1999b). Therefore, it is possible that the soluble pools of abnormal huntingtin molecules are deleterious to the cell, and that aggregate formation may be protective by reducing the level of the abnormal proteins in a soluble phase.

In several models, expression of polyQ-containing polypeptides has been shown to cause cellular stress responses (Chai et al., 1999; Stenoien et al., 1999). Such a response involves induction of heat shock proteins, which were reported to be induced in cells expressing polyQ (Cummings et al., 1998; Chai et al., 1999), and to protect from polyQ-induced neurodegeneration in a mouse model (Cummings, C.J., and H.Y. Zoghbi, personal communication), as well as from apoptosis in *Drosophila* (Kazemi-Esfarjani and Benzer, 2000). Another component of the stress response is activation of stress kinases, which may initiate an apoptotic program (Gabai et al., 1998). PolyQ expression was shown in cell culture to activate the stress kinase c-Jun NH<sub>2</sub>-terminal kinase (JNK), which appears to be essential for apoptosis triggered by such expression (Liu, 1998; Yasuda et al., 1999). Thus, cells accumulating polypeptides with expanded polyQ sequences appear to be constantly exposed to internal "stress," and respond to it by activation of a protective mechanism and/or initiation of the apoptotic program.

Regardless of whether protein aggregation is a protective or a proapoptotic cellular event, it is conceivable that formation of IBs in a cell may be regulated under stressful conditions caused by accumulation of abnormal proteins. One attractive possibility is that IB formation is stimulated by the stress-activated signaling cascade. To test this idea, we focused on a protein kinase, mitogen-activated protein kinase (MAPK) kinase kinase (MEKK)1, which is activated by various stressful treatments (e.g., UV irradiation, DNA-damaging agents, cytokines), and regulates stress-induced MAPK signaling pathways, including JNK, extracellular signal-regulated kinase (ERK), p38, and NF- $\kappa$ B (Kyriakis and Avruch, 1996). MEKK1 is a 196-kD serine-threonine kinase that can associate with, and be activated

by, small GTP-binding proteins (Collins et al., 1996). MEKK1 can also be activated by a caspase-mediated cleavage, resulting in an active 91-kD fragment (Widmann et al., 1998). In this work we studied the effects of MEKK1 on the formation of IBs by polyQ-containing polypeptides and other abnormal proteins.

## Materials and Methods

### Plasmids and Antibodies

DNA constructs for expression of huntingtin, with alternating CAG/CAA repeats of different length, have been described previously (Kazantsev et al., 1999; Preisinger et al., 1999; Steffan et al., 2000). Exon1 sequences with 25 or 104 glutamines were fused in frame with an enhanced green fluorescence protein (GFP) tag at the COOH terminus of each construct (25QP, 104QP). The 47Q construct lacked the proline-rich domain of exon1, and in addition to the GFP tag on its COOH terminus, had a FLAG tag attached to the NH<sub>2</sub> terminus of the huntingtin sequence.

Plasmids encoding either constitutively active or kinase-dead mutant of FLAG-tagged MEKK1 with deleted NH<sub>2</sub>-terminal domain, were a gift of Dr. J. Avruch (Massachusetts General Hospital, Boston, MA). A plasmid encoding GFP-tagged cistic fibrosis transmembrane conductance regulator (CFTR) with a point deletion ( $\Delta$ F508) was a gift of Dr. R. Kopito (Stanford University, Stanford, CA). A plasmid encoding GFP-tagged firefly luciferase was a gift of Dr. R. Day (University of Virginia, Charlottesville, VA). A plasmid encoding constitutively active Raf (Raf-CAAX) was a gift of Dr. D. Stokoe (University of California at San Francisco, San Francisco, CA).

In this study we used antibodies raised against the following: GFP (polyclonal) (CLONTECH Laboratories, Inc.); FLAG epitope (M2),  $\gamma$ -tubulin, and vimentin (Sigma-Aldrich); active-JNK (pTPpY) (Promega); phospho-p38 (Tyr182) and phospho-p42/44 MAPK (polyclonal) (New England Biolabs, Inc.); and heat shock proteins Hsp72 (SPA-810) and Hsp73 (SPA-815) (StressGen Biotechnologies).

### Cell Cultures and Transfection

HeLa human cervical carcinoma cell line, 293 human embryonic kidney (HEK) cell line, and HN33 rat hippocampal neuronal cell line (received from Dr. B. Wainer, Emory University, Atlanta, GA) were grown in DME supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO<sub>2</sub>. EcR-293 cell lines expressing huntingtin–GFP fusions under the control of an inducible promoter have been described previously (Kazantsev et al., 1999). The expression of an integrated gene of the NH<sub>2</sub>-terminal huntingtin fragment containing 300 polyglutamines fused at the COOH terminus with GFP tag was induced by Muristerone A (Invitrogen) according to the manufacturer's protocol.

For transfection, cells were grown in 35-mm dishes to 25–50% confluence, and then washed twice with Opti-MEM 1 (Life Technologies). HeLa cells were transfected for 14–18 h with lipofectamine (Life Technologies) in accordance with the manufacturer's protocol, using 3  $\mu$ l lipofectamine and 1  $\mu$ g of DNA (0.8  $\mu$ g of either 104QP, cistic fibrosis transmembrane conductance regulator CFTR, or luciferase plasmid, and 0.2  $\mu$ g of either MEKK1 plasmid or carrier DNA) per dish. HN33 cells were transfected under similar conditions with the following modifications: cells were transfected for 10 h with 10  $\mu$ l of lipofectamine and 2.5  $\mu$ g of DNA (2.3  $\mu$ g of 104QP plasmid and 0.2  $\mu$ g of either MEKK1 plasmid or carrier DNA) per dish. For an experiment with coexpression of Raf kinase in HeLa cells, cell culture was transfected for 16 h with GenePORTER™ transfection reagent (Gene Therapy Systems) in accordance with the manufacturer's protocol, using 9  $\mu$ l of the reagent and 1.5  $\mu$ g of DNA (0.9  $\mu$ g of 104QP plasmid and either 0.6  $\mu$ g of carrier DNA, or 0.6  $\mu$ g of Raf plasmid, or 0.2  $\mu$ g of MEKK1 plasmid, and 0.4  $\mu$ g of carrier DNA) per dish. 293 cells were transfected for 1.5 h with GenePORTER™ transfection reagent using 18  $\mu$ l of the reagent and 3  $\mu$ g of the plasmid per dish. To end transfection, the dishes were washed once and left in a regular medium as for the subsequent treatments and observations described in the text. Efficiency of transfections was usually in a range of 50–70%.

### Analysis of IB Formation

A Nikon inverted microscope with a 10 $\times$  objective was used to observe IB formation. The number of transfected cells with one or more visible IBs, and

the number of transfected cells without IBs were counted independently in five randomly chosen microscope fields in different areas of a dish. Between 200 and 700 transfected cells were analyzed for each sample. The fractions of IB-containing cells and 95% confidence interval were calculated.

### ***Immunocytochemistry and Confocal Microscopy***

A Leica TCS 4D laser scanning confocal microscope (MKS Lasertechnik) with a 40× oil immersion objective was employed to obtain images of either live transfected cells expressing GFP-tagged proteins (FITC only), or transfected cells after fixation and immunostaining (FITC and TRITC). For immunostaining, cells on a plastic dish were frozen in liquid nitrogen, then treated at -20°C with 100% methanol for 6 min, followed by treatment with a mixture of methanol and acetone (1:1). Thereafter, all procedures were carried out at room temperature. Cells were fixed for 5 min with 4% formaldehyde in PBS, fixed for 5 min in the same solution with 0.5% Triton X-100, rinsed twice with PBS, and incubated overnight with primary antibodies. After thorough washing with PBS, samples were incubated for 3–5 h with rhodamine-conjugated secondary antibody, washed with PBS, and analyzed under the microscope. Primary and secondary antibodies were dissolved in DME supplemented with 50 mg/ml IgG from nonimmunized animals to reduce nonspecific binding.

### ***Preparation of Cell Lysates and Their Analysis***

Cells were washed twice with PBS on a dish, aspirated, and lysed by thoroughly scraping with a plastic scraper in 200  $\mu$ l lysis buffer per 35-mm dish (40 mM Hepes, pH 7.5, 50 mM KCl, 1% Triton X-100, 2 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM  $\beta$ -glycerophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamide, and 5  $\mu$ g/ml each of leupeptin, pepstatin A, and aprotinin). Protein concentration was measured in the lysates, after which they were diluted with lysis buffer to achieve equivalent protein content in all samples. All procedures were performed at 4°C.

For separation of soluble and insoluble fractions, the lysates were subjected to centrifugation at 400 g for 10 min. The pellets were washed once with the lysis buffer, and resuspended in a volume of water equal to the volume of the supernatant. All samples were supplemented with loading SDS-PAGE buffer containing 2% SDS, and were boiled for 3 min before being subjected to immunoblotting. GFP-tagged polyQ polypeptides were visualized with anti-GFP antibody. FLAG-tagged MEKK1 mutants were visualized with anti-FLAG antibody. A working dilution of 1:1,000 was used for all primary antibodies except the phospho-JNK antibody (1:5,000). Secondary antibodies conjugated with peroxidase were visualized with ECL substrates (Amersham Pharmacia Biotech).

In some experiments, the soluble fraction was subjected to further differential centrifugation as described in the text. All obtained samples were treated as described above for the immunoblot analysis.

In some experiments, the soluble fraction was also subjected to gel filtration on 30-ml Superose 6 FPLC column (Amersham Pharmacia Biotech). A 200- $\mu$ l sample was loaded on a column and either 1- or 0.25-ml fractions were collected, precipitated with 10% TCA, washed once with acetone, and dissolved in loading SDS-PAGE buffer for immunoblot analysis.

### ***In Vitro Measurement of MEKK1 Activity***

5  $\mu$ l of either the soluble fraction or the insoluble fraction (resuspended in an equal volume of lysis buffer) was added to a reaction mixture (25  $\mu$ l final volume) containing (final concentration): 25 mM Hepes, pH 7.5, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM  $\beta$ -glycerophosphate, 10 mM MgCl<sub>2</sub>, 2 mM DTT, 15  $\mu$ M ATP, 15  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and 40 ng of recombinant SEK1-GST. The reaction was allowed to proceed for 15 min at 30°C and then stopped by addition of 10  $\mu$ l of loading SDS-PAGE buffer. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and exposed to x-ray film.

## ***Results***

### ***Expression of Active MEKK1 Mutant Enhances Formation of IBs of Mutant Huntingtin***

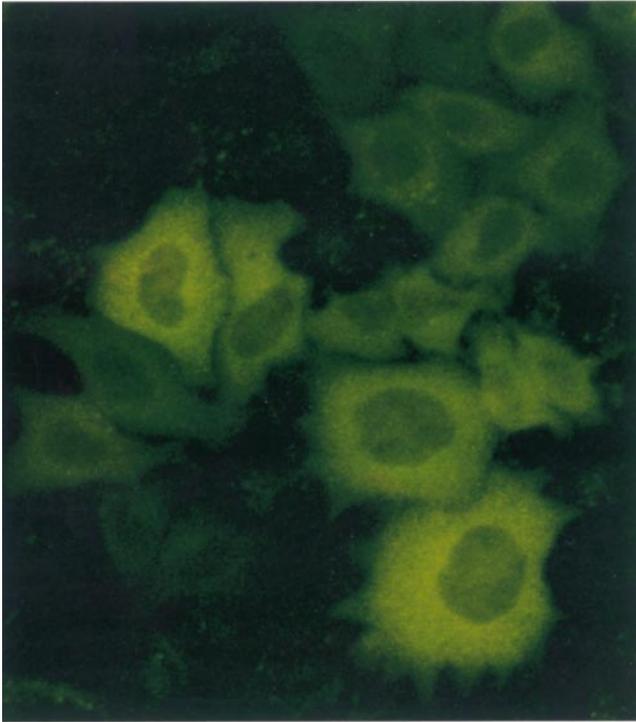
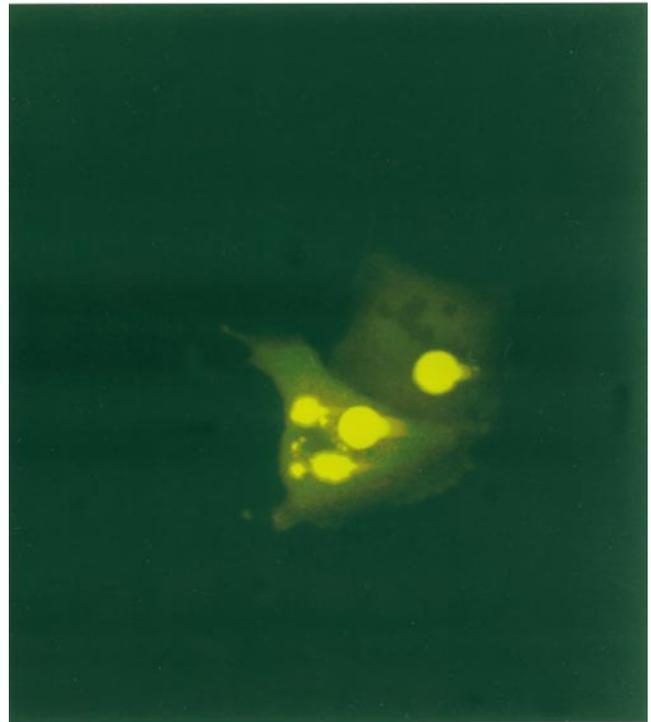
To investigate the possible regulation of IB formation, we studied intracellular aggregation of NH<sub>2</sub>-terminal fragment of huntingtin. Exon1 segment of this protein contains a polyQ domain flanked by 17 residues at the NH<sub>2</sub> terminus and by a proline-rich stretch at its COOH termi-

nus (The Huntington's Disease Collaborative Research Group, 1993). We employed an exon1 sequence including either 25 or 104 glutamine residues, which represent normal and mutant forms, respectively. COOH termini of these constructs were fused with the GFP sequences, resulting in 25QP and 104QP polypeptides. When such constructs were transiently transfected into mammalian cells, GFP allowed detection of expression and localization of the chimerical polypeptides by fluorescent microscopy. 25QP appeared completely soluble, whereas a large fraction of cells expressing 104QP contained bright fluorescent IBs (Fig. 1, A and B).

To investigate the effects on protein aggregation of MEKK1, an important mediator of multiple stress-activated signaling pathways, HeLa cells were transfected either with 104QP alone or together with a plasmid encoding a truncated constitutively active form of MEKK1 (MEKK1 $\Delta$ ). Introduction of the MEKK1 $\Delta$  led to a large increase in the fraction of cells that form IBs. Indeed, after 16 h of transfection with 104QP alone, the fraction of HeLa cells that formed one or more IB detectable by the fluorescence microscopy was usually far less than 5%, whereas in a culture cotransfected with active MEKK1 no less than 15–20% of cells contained IBs. In the course of the next 48 h the fraction of transfected cells with detectable IBs grew in both cultures; however, the MEKK1 $\Delta$ -dependent increase in IB formation was sustained (Fig. 2 A). It is noteworthy that in the course of the experiment, neither expression of 104QP nor coexpression of MEKK1 $\Delta$  caused any significant change in viability of the transfected cells (not shown). Also, MEKK1 $\Delta$  did not cause formation of IBs from the normal size polyQ domain, 25QP (not shown).

The stimulating effect of MEKK1 on aggregation of 104QP was also observed with the hippocampal HN33 cell line (Fig. 2 B), as well as with 293, H9c2, and CHO cells (not shown). Although the control levels of IB formation observed in these different cell lines varied, expression of active MEKK1 always led to an increase in IB formation.

To avoid potential artifacts due to simultaneous cotransfection with two different plasmids, we employed the 293 cell line stably transfected with a polyQ-encoding gene under the control of the muristerone-inducible promoter. 293 cells expressed the 300Q-GFP fusion (300Q), which lacks the proline-rich region of exon1. Our experiments indicated that this region is not critical for either aggregation of polyQ polypeptides or the stimulatory effect of MEKK1 on such aggregation (not shown). 300Q was induced with muristerone for 24 h, then cells were transiently transfected with MEKK1 $\Delta$  gene and incubated in the presence of muristerone for 24 h. Because in this experiment all cells accumulated 300Q but only a few of them formed IBs, it was technically difficult to count a fraction of IB-containing cells. Instead, we counted the number of IBs in five randomly chosen microscopic fields. The number of cells on a dish and cellular levels of 300Q were similar in control cultures and cultures expressing MEKK1 $\Delta$  (not shown). As seen in Fig. 2 C, the expression of MEKK1 $\Delta$  led to more than a twofold increase in IB formation above levels in the mock-transfected cells. Because the efficiency of transient transfection under these conditions was ~50% (not shown), MEKK1 $\Delta$  must have been expressed in only half of the cells, and therefore its effect

**A****25QP****B****104QP**

**Figure 1.** Aggregation of 104QP in HeLa cells viewed by confocal microscopy. (A) Cells expressing 25QP construct; (B) cells expressing 104QP construct.

on IB formation was underestimated by at least twofold in these experiments.

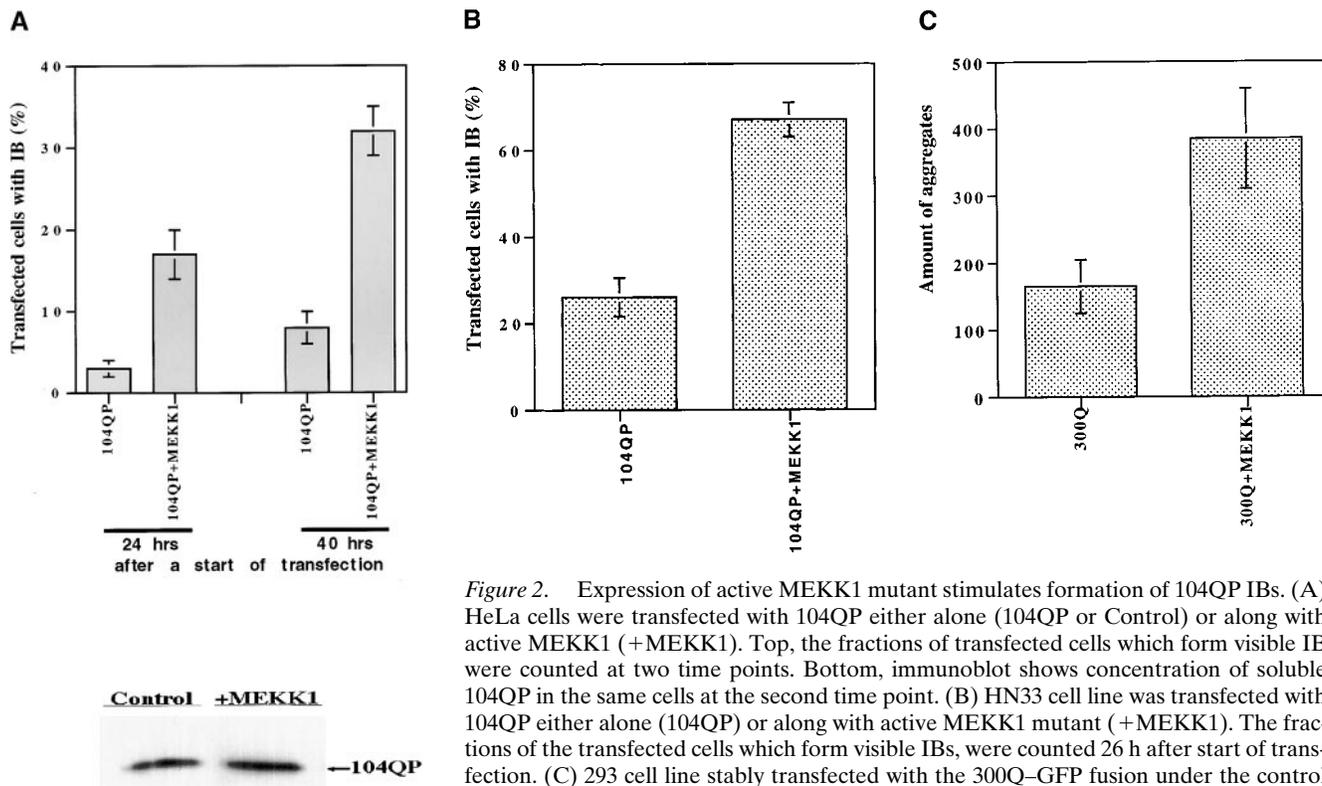
To test if the stimulation of 104QP aggregation requires the kinase activity of MEKK1, we investigated if a kinase-dead mutant of MEKK1 can also stimulate formation of IBs, when coexpressed with 104QP. In contrast to MEKK1 $\Delta$ , the kinase-dead mutant failed to significantly stimulate 104QP aggregation (Fig. 3 A, top). Note that the cellular levels of the kinase-dead mutant were equal to, or even greater than, the level of MEKK1 $\Delta$  (Fig. 3 A, bottom).

We also tested whether a protein kinase Raf, which regulates MAPK cascades at the same level as MEKK1 (Cobb and Goldsmith, 1995; Schlesinger et al., 1998), can similarly stimulate formation of IBs. HeLa cells were transfected (Materials and Methods) with 104QP expression vector together with 0.6  $\mu$ g of activated Raf expression vector (as a positive control we cotransfected cells with 0.2  $\mu$ g of MEKK1 $\Delta$  expression vector). Under these conditions the expression of activated Raf did not cause a significant increase in IB formation (Fig. 3 B, top), whereas cells cotransfected with MEKK1 formed aggregates 2.5–3 times more often than cells expressing just 104QP. Note that expression of active forms of Raf or MEKK1 led to approximately equal activation of ERK in transfected cells (Fig. 3 B, bottom).

#### ***MEKK1 Reduces the Solubility of Polyglutamine-containing Polypeptides***

IB formation must involve redistribution of 104QP molecules to the insoluble fraction. Therefore, we studied the effect of MEKK1 on the solubility of 104QP. HeLa cells transfected with 104QP were lysed in a buffer containing Triton X-100, and were then subjected to differential centrifugation. The first centrifugation was for 10 min at 400 g, then the supernatant was centrifuged at 15,000 g for 10 min, and finally the resulting supernatant was subjected to centrifugation at 380,000 g for 40 min. The resulting fractions were dissolved in buffer containing 2% SDS. We found that 104QP, detected by immunoblotting with anti-GFP antibody, was present in the lysate in two distinct fractions: the 400-g pellets, and the 380,000-g supernatant (Fig. 4 A). A similar experiment was done with polyQ of normal length, and all 25QP were found in the 380,000-g supernatant (not shown). Therefore, the appearance of polyQ fusion proteins in the particulate fraction correlates with the tendency to form IBs.

To investigate the formation of IBs, the 104QP-containing supernatant from the first, low speed centrifugation was subjected to gel filtration on Superose 6. Surprisingly, almost all 104QP migrated as one peak of  $\sim$ 350 kD (Fig. 4 B), which far exceeds the molecular mass of 104QP monomer ( $\sim$ 45 kD). These data indicate that soluble 104QP ex-



**Figure 2.** Expression of active MEKK1 mutant stimulates formation of 104QP IBs. (A) HeLa cells were transfected with 104QP either alone (104QP or Control) or along with active MEKK1 (+MEKK1). Top, the fractions of transfected cells which form visible IB were counted at two time points. Bottom, immunoblot shows concentration of soluble 104QP in the same cells at the second time point. (B) HN33 cell line was transfected with 104QP either alone (104QP) or along with active MEKK1 mutant (+MEKK1). The fractions of the transfected cells which form visible IBs, were counted 26 h after start of transfection. (C) 293 cell line stably transfected with the 300Q-GFP fusion under the control of the muristerone-inducible promoter was incubated with muristerone for 24 h. Cells

were then transfected with either MEKK1 $\Delta$  gene (300Q+MEKK1) or carrier DNA alone (300Q) and further incubated in the presence of muristerone for an additional 24 h. The number of IBs in five randomly chosen microscope fields was counted.

ists as either a homooligomer of a particular size or a discrete complex with certain cellular proteins. This observation suggests that upon IB formation, polyQ polypeptides do not generate oligomers of various molecular masses (distributed over a wide range of fractions rather than concentrated in one peak). The 25QP were also found exclusively in a high molecular mass peak (not shown).

We did not observe a change in the elution of the 104QP peak in the lysates of cells expressing MEKK1 $\Delta$  (not shown), suggesting that formation of the complex is not regulated by this kinase. On the other hand, transfection with MEKK1 $\Delta$  led to a redistribution of 104QP to 400-g pellets (insoluble fraction) (Fig. 5 A, top). Importantly, transfection with the kinase-dead mutant of MEKK1 did not significantly affect the solubility of 104QP (Fig. 5 A), indicating that the kinase activity of MEKK1 is critical for its stimulation of 104QP redistribution to the insoluble fraction.

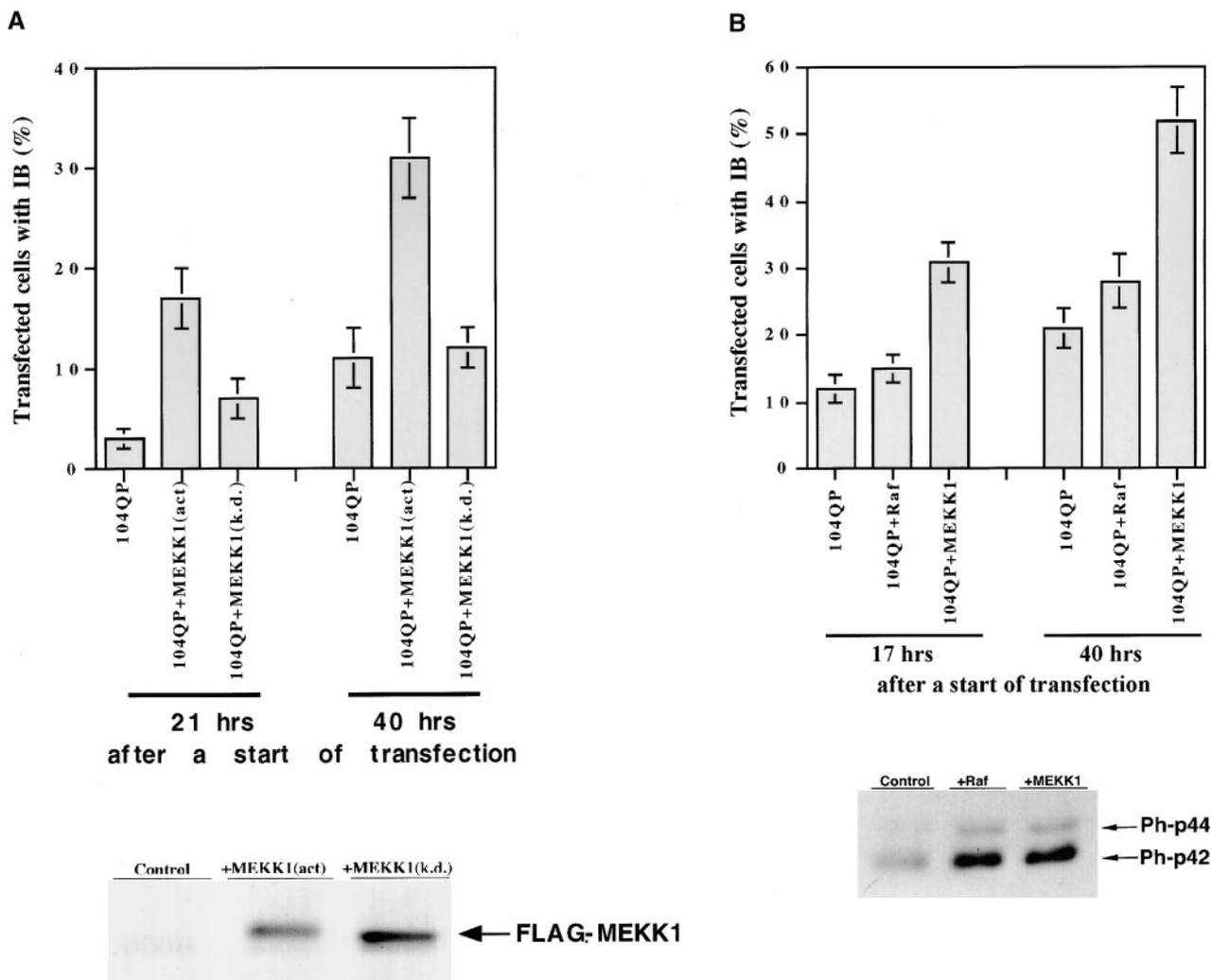
The 104QP found in the 400-g pellets represents polypeptides that are present in IBs and/or associated with large nonmembranous cellular components, e.g., cytoskeleton (as cell lysates were prepared in the presence of the detergent, which should dissolve membranous structures). Seemingly, IB formation correlates with redistribution of polyQ polypeptides to the insoluble fraction: both processes depend on the length of polyQ, they have similar time courses (not shown), and they are stimulated by MEKK1 $\Delta$ .

Surprisingly, in spite of the apparent correlation, stimulation by MEKK1 of redistribution of polyQ polypeptides to the insoluble fraction could be dissociated from the stimulation of IB formation. This was demonstrated with a fu-

sion polypeptide 47Q consisting of the glutamine-rich part of huntingtin's exon1 with 47 glutamines fused to GFP (see Materials and Methods). As found with 25QP and 104QP, the soluble 47Q polypeptide (from 400-g supernatant) migrated as a single peak on a Superose 6 column (not shown). This polypeptide did not form visible IBs when expressed in HeLa cells, and cotransfection with MEKK1 $\Delta$  did not lead to IB formation (not shown). However, MEKK1 $\Delta$  expression caused a large increase in the fraction of 47Q associated with 400-g pellets (multiple bands on the immunoblot of 47Q in the pellet probably represent ubiquitinated forms of the polypeptide) (Fig. 5 B). It should be noted that in cells expressing active MEKK1, a small fraction of otherwise completely soluble 25QP was also detected in the pellet (not shown). Therefore, MEKK1 stimulates redistribution of polyQ polypeptides into the insoluble fraction, which may or may not result in formation of visible IBs, depending of the length of polyQ.

#### **104QP IBs Colocalize with MEKK1 in Distinct Cytosolic Structures**

The fact that MEKK1 $\Delta$  stimulates recruitment of polyQ-containing polypeptides to the particulate fraction suggests that this kinase may associate with 104QP IB. Indeed, a large fraction (sometimes >50%) of MEKK1 $\Delta$  can be found in 400-g pellets (Fig. 5 A, bottom). Such association of MEKK1 $\Delta$  with the insoluble fraction can be observed both in cells that coexpress 104QP (Fig. 5 A) and cells that express MEKK1 $\Delta$  alone (not shown), indicating that the affinity to particulate fractions is an intrinsic trait



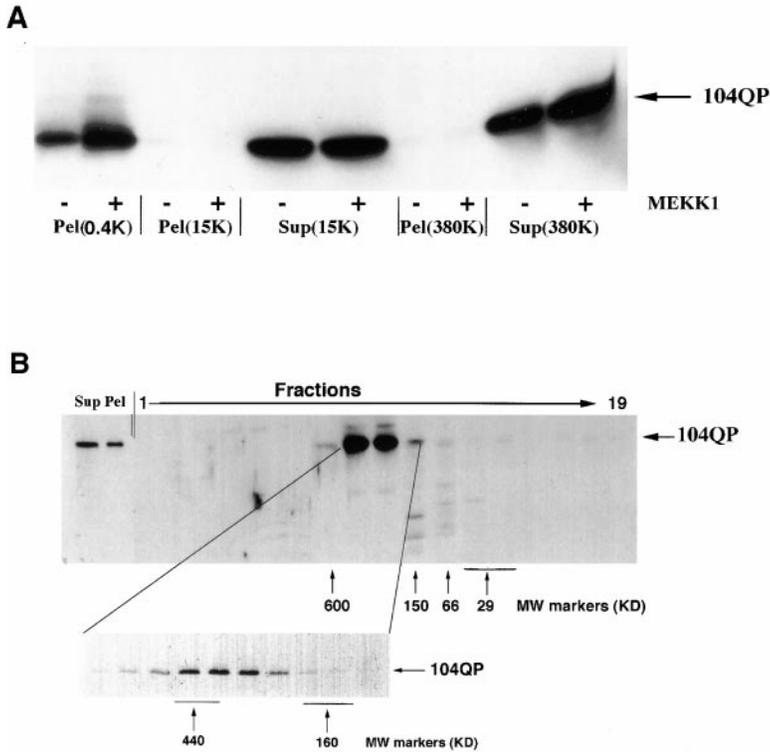
**Figure 3.** Stimulation of 104QP IB formation depends on the MEKK1 activity. (A) HeLa cells were transfected with 104QP either alone (104QP or Control) or along with active MEKK1 mutant (+MEKK1 [act]), or along with kinase-dead MEKK1 mutant (+MEKK1 [k.d.]). Top, the fractions of transfected cells which form visible IBs were counted at two time points. Bottom, content of MEKK1 mutants in a soluble fraction of the same cells. Immunoblotting with anti-FLAG antibody. (B) HeLa cells were transfected with 104QP either alone (104QP or Control) or along with either active MEKK1 mutant (+MEKK1) or active Raf (+Raf) (to increase the amount of Raf DNA in transfection, it was done differently [see Materials and Methods]). Top, the fractions of transfected cells which form visible IBs were counted at two time points. Bottom, activation of ERK by transfected MEKK1 or Raf mutants in a soluble fraction of the same cells. Immunoblotting with anti-phospho-ERK (p42/44 MAPK) antibody.

of active MEKK1. It is noteworthy that a much lower portion of the kinase-dead mutant, which did not stimulate IB formation, was found in 400-g pellets (Fig. 5 A, bottom). Therefore, the kinase activity appears to be critical for association of MEKK1 with the insoluble fraction.

To test whether the MEKK1 $\Delta$  that was associated with the insoluble fraction was inactive or retained its activity, we resuspended the pellet in lysis buffer and assayed it along with the soluble fraction for MEKK1 activity. Purified recombinant SEK1, MEKK1 substrate, was added to both fractions, and the ability of MEKK1 $\Delta$  to phosphorylate this protein was tested in the presence of [ $\gamma$ - $^{32}$ P]ATP. Strong phosphorylation of SEK1 was observed in both the 400-g pellet and the supernatant from lysates of cells transfected with MEKK1 $\Delta$  (Fig. 6). The apparently lower MEKK1 activity in the insoluble fraction in comparison with the supernatant reflects, most probably, incom-

plete accessibility of MEKK1 $\Delta$  to its substrate in the resuspended pellet. The assayed activity was specific to MEKK1, because we did not observe phosphorylation of SEK1 in the lysates of cells transfected with 104QP alone (Fig. 6, MEKK1 [-]). Therefore, MEKK1 $\Delta$  is active in the insoluble fraction.

MEKK1 was previously shown to localize at distinct structures in a cell (Christerson et al., 1999). To study the role of such structures in formation of 104QP-containing IBs, we used an immunolocalization approach. FLAG-tagged MEKK1 $\Delta$  was cotransfected with 104QP. Cells were probed with anti-FLAG antibody and further treated with a rhodamine-labeled secondary antibody. In accord with previous reports, MEKK1 $\Delta$  was located at discrete spotted cytosolic structures (Fig. 7 A, red). Such structures were present even in the cells, which did not form 104QP-containing IBs, indicating that localization into these



**Figure 4.** 104QP is present in transfected cells in two distinct forms: supernatant (Sup) and pellet (Pel). Immunoblots are shown. (A) Differential centrifugation. HeLa cells were transfected with 104QP either alone (–MEKK1) or along with active MEKK1 (+MEKK1). 40 h after the end of transfection cells were collected, cell lysates were diluted to equalize total protein concentration and subjected to consecutive centrifugations: 10 min at 400 g (0.4K), 10 min at 15,000 g (15K), and 40 min at 380,000 g (380K). (B) Soluble 104QP is present in a complex of a distinct size. HeLa cells transfected with the 104QP construct were lysed and subjected to low speed centrifugation at 400 g for 10 min (Sup and Pel). The supernatant was loaded onto a Superose 6 FPLC column and gel filtration was performed in lysis buffer (see Materials and Methods). Fractions were collected (numbering starts upon elution of a void volume), concentrated by TCA precipitation, and analyzed by immunoblotting. Inset shows the results of the gel filtration with the four-time decreased fraction size.

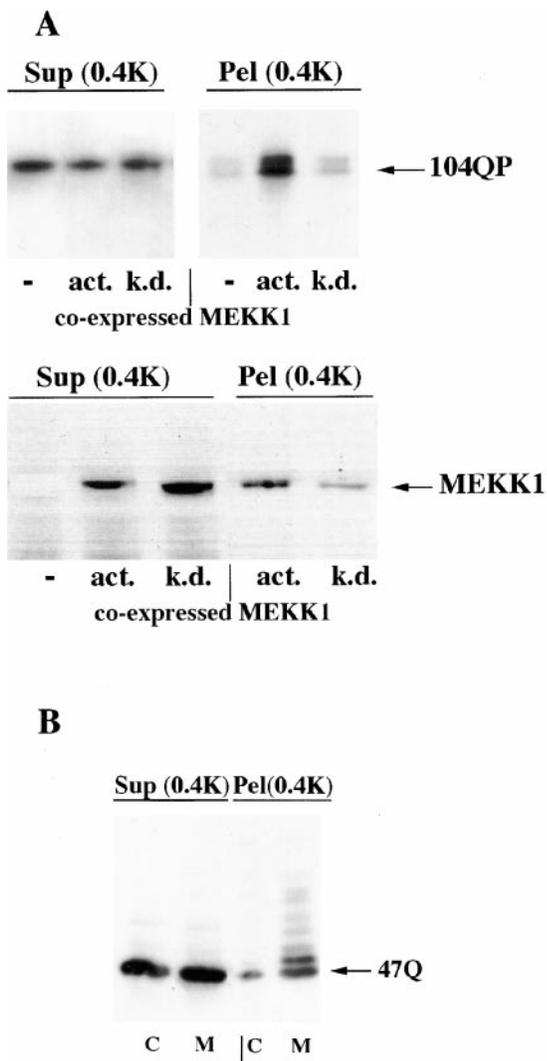
structures is an intrinsic trait of active MEKK1, independent of 104QP. These data are in agreement with the already mentioned ability of MEKK1Δ to associate with insoluble fraction independently of 104QP. Double fluorescence of rhodamine and GFP, detected with confocal microscopy, showed that all 104QP-containing IBs colocalized (Fig. 7 A, yellow) with the structures formed by MEKK1Δ, suggesting that MEKK1 may be involved in the organization of sites of polyQ aggregation.

### Similarities and Differences between Polyglutamine-containing IB and Aggresome

IBs formed by polyQ polypeptides are reminiscent of aggresomes formed by certain abnormal proteins (Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999). Therefore, we investigated whether IBs formed with polyQ polypeptides are indeed similar to aggresomes, which have been reported to localize to centrosomes, to be encircled by vimentin filaments, and to contain molecular chaperones and components of the ubiquitin–proteasome degradation machinery. To investigate whether the polyQ-containing IBs are of similar composition and origin, we again used an immunolocalization approach. Cells transfected with 104QP were immunostained with various antibodies, and further treated with a rhodamine-labeled secondary antibody. To define the location of the centrosome, we used immunostaining with anti-γ-tubulin antibody, the commonly used centrosome marker (Oakley and Oakley, 1989; Diktenberg et al., 1998). Because the centrosomes in HeLa cells are diffused and cannot be clearly detected with anti-γ-tubulin antibody (not shown), we used HEK 293 cells where centrosomes are more condensed and could be visualized by immunostaining as de-

scribed previously (Kopito, 1999; Wigley et al., 1999). Double fluorescence clearly indicated that 104QP IBs colocalized with γ-tubulin-containing structures (Fig. 7 B, left). As seen in Fig. 7 B, the centrosomes in IB-containing cells appeared larger than those in cells without IBs, which is consistent with the prior observation that centrosome size increases with aggresome formation (Johnston et al., 1998; Wigley et al., 1999). Furthermore, like aggresomes, the polyQ-containing IB was surrounded by a network of vimentin filaments in HeLa cells (Fig. 7 B, right). Moreover, like the aggresome, IBs with 104QP were associated with both constitutive and inducible members of the Hsp70 protein family, Hsc73 and Hsp72 (Fig. 7 C).

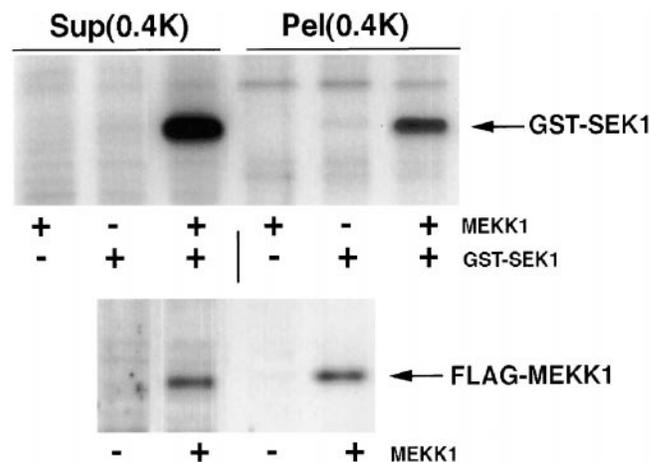
On the other hand, there is an important difference between formation of aggresomes and formation of polyQ IBs, because in HeLa cells the latter process is apparently independent of microtubules. To test for involvement of microtubules in the IB formation we employed nocodazole, which disrupts the microtubular network and has been reported to prevent aggresome formation (Johnston et al., 1998; Garcia-Mata et al., 1999). HeLa cells transfected with 104QP were either treated for 6 h with 10 μg/ml of nocodazole or left untreated. Nocodazole treatment did not block formation of IBs during this time, but rather potentiated it (Fig. 8). No inhibition of IB formation by nocodazole was seen either in the presence or absence of MEKK1Δ (Fig. 8). Furthermore, nocodazole did not increase the fraction of cells with multiple IBs (not shown). Similarly, treatment of transfected HeLa cells with 40 μM cytochalasin B, which disrupts actin filaments, did not cause any decrease in basal and MEKK1-induced formation of IBs (Fig. 8). Thus, both basal and MEKK1-stimulated formation of polyQ-containing IBs do not depend on microtubules and actin filaments in HeLa cells.



**Figure 5.** Expression of active MEKK1 mutant causes association of polyQ polypeptides with the insoluble fraction. Transfected HeLa cells were lysed and subjected to low-speed centrifugation at 400 g for 10 min. Immunoblots are shown. Pel(0.4K), pellet; Sup(0.4K), supernatant. (A) Cells were transfected with either 104QP alone (–) or along with active MEKK1 mutant (act.) or along with kinase-dead mutant (k.d.). Samples were collected 20 h after the end of transfection. Top, samples probed with anti-GFP antibody for 104QP. Bottom, samples probed with anti-FLAG antibody for MEKK1 mutants. (B) Cells were transfected with either 47Q alone (C) or along with active MEKK1 (M). Samples were collected 25 h after the end of transfection. In this immunoblot a pellet fraction was resuspended in half of the original volume.

### Stressful Treatments Can Stimulate 104QP IB Formation

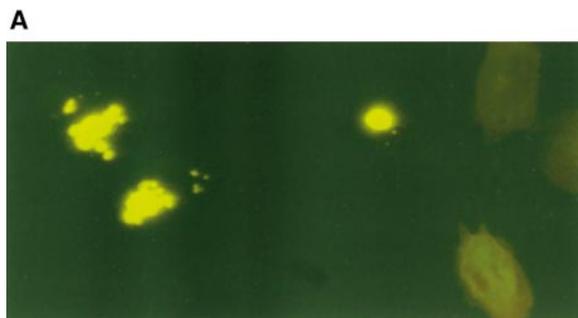
The fact that MEKK1Δ is able to stimulate polyQ aggregation leads to an interesting possibility: that the stressful conditions that activate MEKK1 may facilitate formation of polyQ IBs. In accordance with a previous report (Wyttenbach et al., 2000), we observed that heat shock strongly stimulates formation of polyQ-containing IBs (not shown), but this may be due to a direct denaturing effect of heat treatment on the polyQ polypeptide. Therefore, it would be more informative to test for the effect of



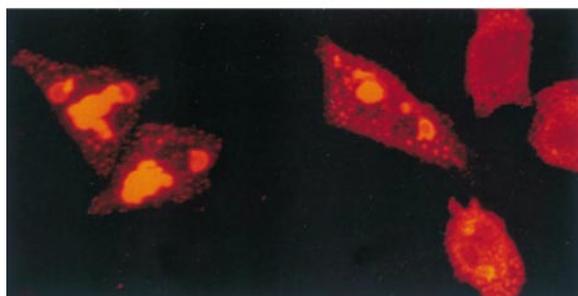
**Figure 6.** MEKK1Δ in insoluble fraction is active. HeLa cells were transfected with 104QP either alone (– MEKK1) or along with active MEKK1 (+MEKK1). 20 h after the end of transfection cells were collected, and soluble and insoluble fractions were separated by centrifugations (10 min at 400 g): Sup(0.4K), supernatant; Pel(0.4K), pellet. Pellets were resuspended in lysis buffer and both fractions were tested for their ability to phosphorylate recombinant GST-SEK1. The autoradiogram shows phosphorylation of SEK1 in the samples (–GST-SEK1: no substrate was added to the reaction) (top). The immunoblot with anti-FLAG antibody shows distribution of FLAG-tagged active MEKK1 between soluble and insoluble fractions (bottom).

MEKK1-activating stresses that do not cause protein damage (e.g., UV irradiation). HeLa cells transiently transfected with 104QP were irradiated with 150 J/m<sup>2</sup> UV-C. As seen in Fig. 9 A, IB formation was strongly increased by this treatment. Moreover, UV irradiation caused a significant redistribution of 104QP into the insoluble fraction as was seen with transformation of active MEKK1 (Fig. 9 B). Treatment of transfected HeLa cells with a DNA-damaging agent cisplatin, another stimulus known to activate MEKK1, also stimulated 104QP IB formation (Fig. 9 C).

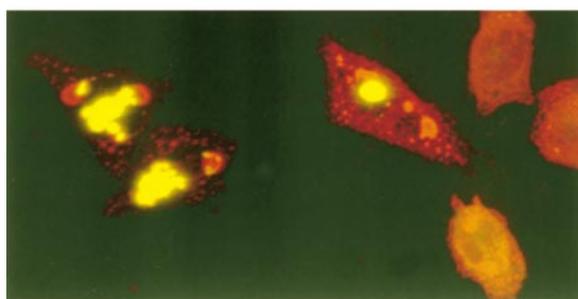
The stressful stimuli are known to cause apoptosis. Indeed, a fraction of the cells died as a result of treatment with either cis-platinum or UV irradiation. There is a possibility that activation of a stress-signaling pathway could lead to enhanced toxicity of nonaggregated 104QP resulting in a cell death and hence in a selective survival of cells with aggregates. However, this model cannot explain the stimulation of IB formation by the recombinant active MEKK1 because, as mentioned above, its coexpression with 104QP did not cause detectable toxicity. There is also a possibility that the stimulation of IB formation by the stressful treatments could result from some late apoptotic event in dying cells. To address this question, we studied if stimulation of IB formation by UV irradiation is affected by general caspase inhibitors. The caspase inhibitor Z-VAD protected cells from apoptosis caused by UV irradiation, but did not inhibit the enhancement of aggregation (not shown). Moreover, continuous exposure of 104QP-transfected HeLa cells to the caspase inhibitors Z-VAD or DEVD did not reduce the MEKK1-induced stimulation of IB formation, indicating that activation of caspases is not critical for this stimulation. Intriguingly, 104QP aggregation was greater in the cells with the caspase inhibitors (Fig.



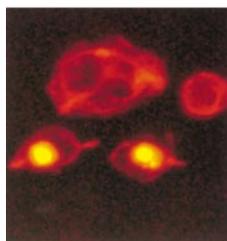
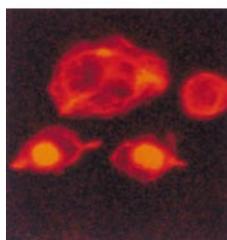
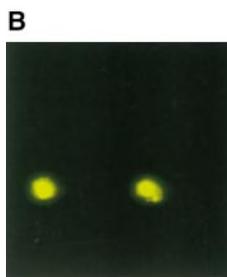
**104QP (GFP)**



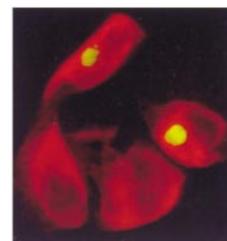
**MEKK1 (FLAG)**



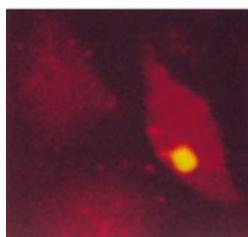
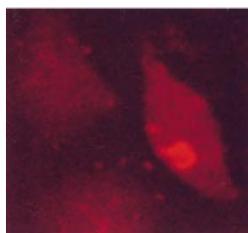
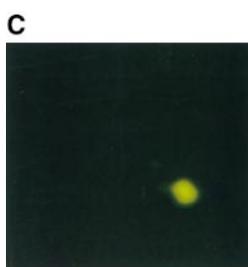
**Merge**



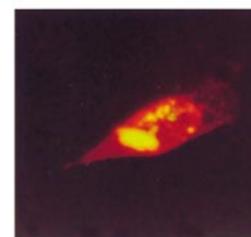
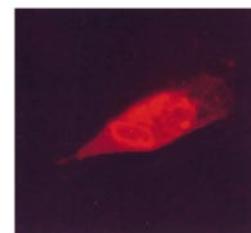
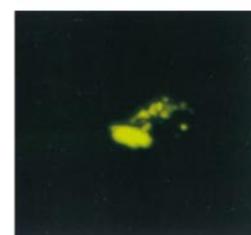
**$\gamma$ -tubulin**



**vimentin**

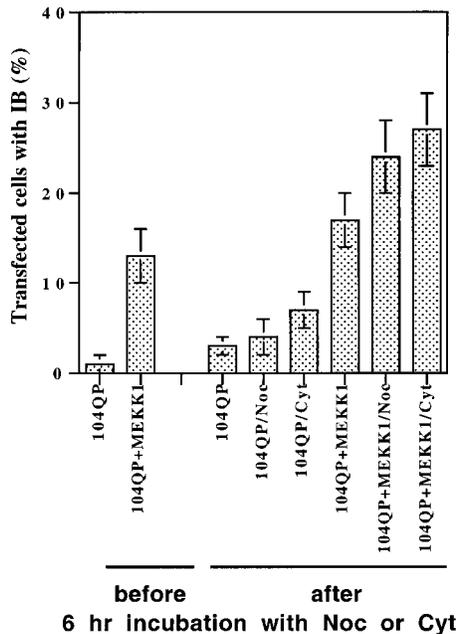


**Hsp73**



**Hsp72**

**Figure 7.** Colocalization of 104QP IB with active MEKK1 and cellular proteins. 24 h after the end of transfection HeLa cells were fixed in a way that mainly retained insoluble structures (Materials and Methods), including 104QP IB (green, top row). Samples were probed with antibodies to various proteins (red, middle row) to study their colocalization (yellow, bottom row) with 104QP. (A) Anti- $\gamma$ -tubulin and antivimentin antibodies. (B) Anti-FLAG antibody to localize FLAG-tagged MEKK1 active mutant. (C) Anti-Hsp73 (constitutive member of the Hsp70 family) and anti-Hsp72 (inducible member of the Hsp70 family) antibodies.



**Figure 8.** Disruption of microtubules or actin filaments does not abrogate stimulation of 104QP IB formation by MEKK1. HeLa cells were transfected with 104QP construct either alone (104QP) or along with active MEKK1 (104QP+MEKK1). After the end of transfection some cells were treated with 10  $\mu$ g/ml of nocodazole (Noc) to disrupt microtubules, or with 40  $\mu$ M cytochalasin B (Cyt) to disrupt actin filaments. The fractions of transfected cells which form visible IBs were counted either before or 6 h after the start of treatments.

9 D). This stimulatory effect was seen both in the presence and in the absence of MEKK1, suggesting that there is a caspase-regulated pathway influencing IB formation.

MEKK1 may exert its action on polyQ aggregation via stimulation of one of the stress-activated signaling pathways. Indeed, it is well documented that MEKK1 may activate JNK, p38, and ERK/ MAP-related kinases (Kyriakis and Avruch, 1996; Schlesinger et al., 1998). There are also reports that this kinase is a potential activator of NF- $\kappa$ B (Lee et al., 1997, 1998). However, it appears that none of these pathways is involved in MEKK1 $\Delta$ -induced stimulation of polyQ aggregation. Indeed, with 293 cells, where MEKK1 $\Delta$  expression activated cellular JNK, p38, or ERK, their active forms did not associate with the insoluble fraction, in contrast to MEKK1 (not shown). Furthermore, pharmaceutical inhibitors of either ERK (U0126), p38 (SB203580), or NF- $\kappa$ B (aspirin) pathways did not significantly reduce MEKK1 $\Delta$ -stimulated formation of 104QP-containing IBs (not shown). Moreover, in the HeLa cell line chosen for this study while stimulating IB formation, expression of MEKK1 $\Delta$  did not cause any detectable activation of JNK (not shown). These data suggest that either MEKK1 directly phosphorylates and activates a component necessary for IB formation, or it acts via a yet unknown signaling pathway.

#### **MEKK1 Stimulates Aggregation of Various Proteins**

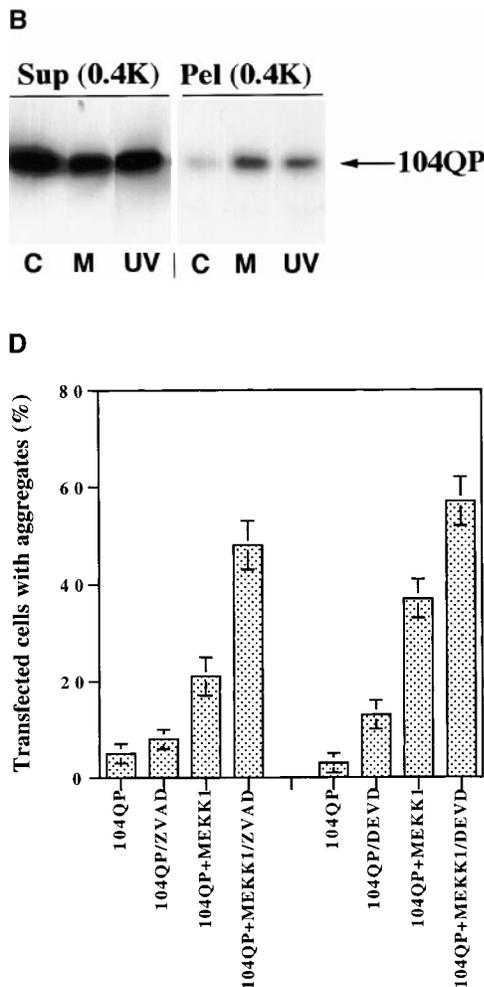
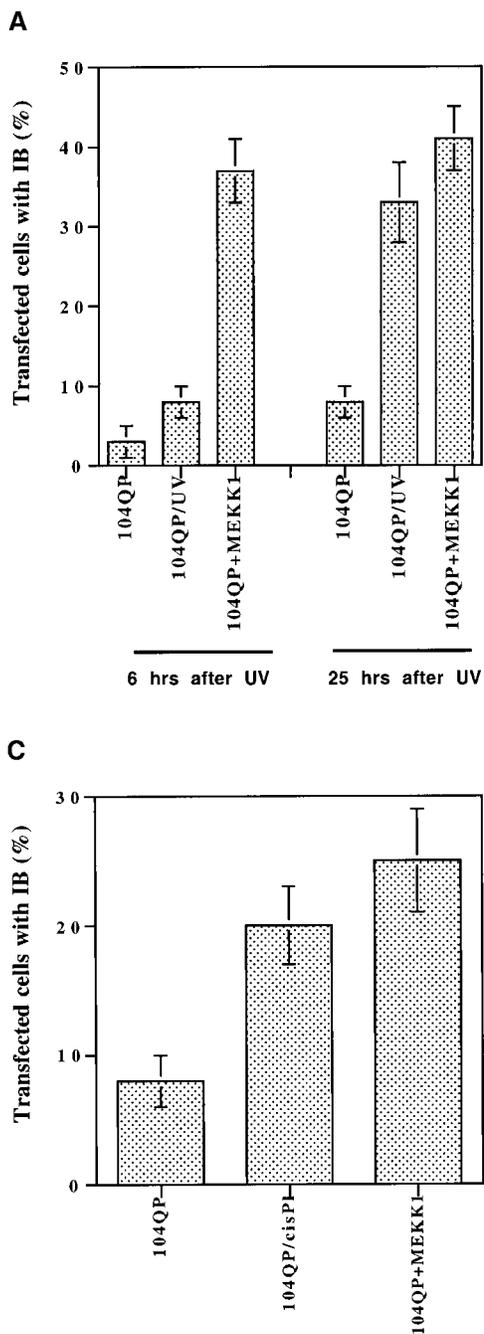
How general is the ability of MEKK1 to stimulate IB formation? To address this question we studied the influence

of this kinase on the aggregation of a polypeptide, which lacks a polyQ domain, a CFTR. The ability of this protein to fold properly is drastically reduced by deletion of a single amino acid (F508), which leads to its rapid proteasome-dependent degradation. If degradation is blocked by a proteasome inhibitor, this polypeptide accumulates and forms an aggresome (Johnston et al., 1998). Transient transfection of HeLa cells with a gene of CFTR fused to GFP led to its accumulation at a very low level in a small fraction of cells. The fluorescence was homogeneous and no IBs were seen (Fig. 10, top, Control). Overnight incubation of transfected cells in the presence of 5  $\mu$ M of the proteasome inhibitor MG132 strongly increased accumulation of CFTR. In a large fraction of fluorescent cells IBs were clearly detectable (Fig. 10, top, MG132). In contrast to polyQ-containing IBs, these IBs were less compressed and often formed a crescent-shaped structure surrounding the nucleus. Cotransfection of CFTR cells with MEKK1 $\Delta$  had an effect similar to that of inhibition of the proteasome activity, i.e., the number of fluorescent cells detectable under a microscope increased, and in most of them IB-like structures were formed (Fig. 10, top, MEKK1).

We also investigated aggregation of a firefly luciferase. This protein is normally soluble, but easily aggregates in cells exposed to heat shock (Michels et al., 1995). To test for the effect of MEKK1 $\Delta$  on luciferase aggregation we employed a GFP-luciferase fusion protein (Day et al., 1998). When transiently expressed in HeLa cells, this fusion polypeptide does not aggregate (Fig. 10, bottom, Control) except in some dead cells (Fig. 10, bottom, Dead cell). Heat shock of cells at 44°C for 30 min followed by a 15-h recovery period led to appearance of luciferase IBs in living cells (Fig. 10, bottom, Heat Shock). To our surprise, cotransfection with MEKK1 also led to aggregation of luciferase without any additional stress (Fig. 10, bottom, MEKK1). These data demonstrate that the effect of MEKK1 on IB formation is not limited to proteins with polyQ extensions.

#### **Discussion**

Here we demonstrate that formation of IBs by polyQ-containing polypeptides as well as other abnormal proteins is regulated by MEKK1, a kinase that activates several stress kinase cascades. This finding is unexpected, as protein aggregation in a cell has been viewed as a spontaneous, energy-independent process that could easily be observed in a test tube with a solution of purified protein. For the same reasons, protein folding was previously viewed as a spontaneous, energy-independent process until it became evident that cells have a very complicated chaperone machinery to facilitate proper folding, which may consume hundreds of ATP molecules per one folded polypeptide chain (Horwich et al., 1999; Agashe and Hartl, 2000; Feldman and Frydman, 2000). Similarly, it appears that protein aggregation in the cell is an active process that involves cellular components. Some features of this process have already been described for several abnormal polypeptides, including specific localization of protein inclusions at the centrosome in the cytosol (Anton et al., 1999; Garcia-Mata et al., 1999; Vidair et al., 1999; Wigley et al., 1999; Fabunmi et al., 2000) or promyelocytic leukemia oncogenic domains



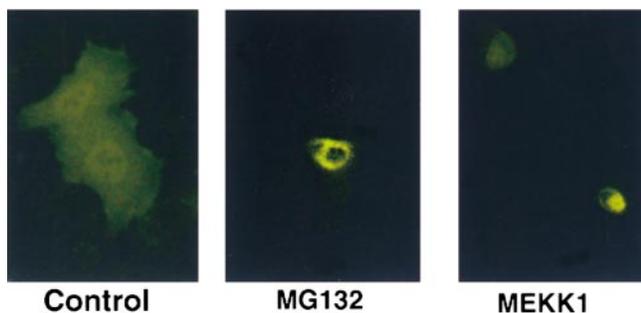
**Figure 9.** Stresses and caspase inhibitors stimulate aggregation of 104QP. HeLa cells were transfected with 104QP either alone (104QP or C) or along with active MEKK1 (104QP + MEKK1 or M). (A) Cells after transfection were either UV-irradiated (150 J/m<sup>2</sup>) 104QP/UV or left untreated and the fractions of transfected cells which form visible IB, were counted at two time points. (B) Immunoblot of soluble supernatant (Sup[0.4K]) and insoluble pellet (Pel[0.4K]) fractions of the same (see Fig. 9 A) transfected cells collected at the second time point. (C) Cells after transfection were either exposed for 12 h to 40  $\mu$ M cisplatin (104QP/cisPI) or left untreated and the fractions of transfected cells which form visible IBs were counted. (D) Cells marked 104QP/ZVAD from the start of transfection were exposed to 100  $\mu$ M ZVAD and the fractions of transfected cells which form visible IBs were counted 17 h after the start. Cells marked 104QP/DEVD from the start of transfection were exposed to 100  $\mu$ M DEVD and the fractions of transfected cells which form visible IBs were counted 21 h after the start.

in the nucleus (Anton et al., 1999), microtubule-dependent transport of microaggregates to the centrosome (Johnston et al., 1998; Garcia-Mata et al., 1999), and recruitment of chaperones and components of the ubiquitin-proteasome pathway (Anton et al., 1999; Garcia-Mata et al., 1999; Vidair et al., 1999; Wigley et al., 1999; Fabunmi et al., 2000). Our findings indicate that protein aggregation in a cell is a regulated process.

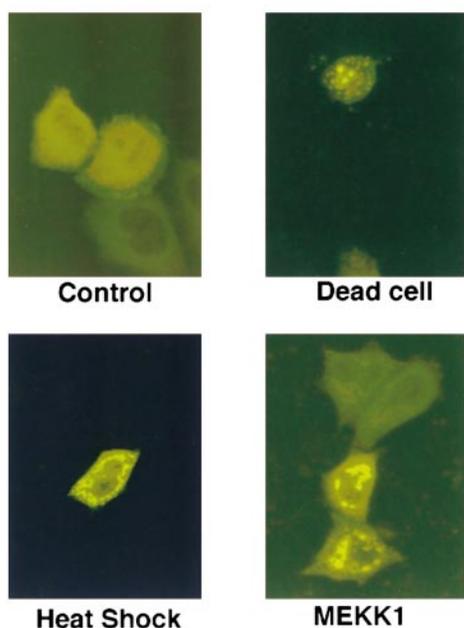
Enhancement of aggregate formation by MEKK1 suggests that protein aggregation may be regulated by stressful stimuli. Many stressful treatments, e.g., heat shock and oxidative stress, cause protein damage. Therefore, enhance-

ment of protein aggregation by these stresses, which has been well documented (Kampinga, 1993; Kabakov and Gabai, 1994), could be explained by direct effects on unfolding of the aggregated polypeptides. The novel possibility is that activation of stress-signaling pathways may also contribute to the stimulation of aggregation by protein-damaging stresses. Furthermore, we demonstrated here that stresses that do not cause direct protein damage (e.g., DNA-damaging agents, UV irradiation) can also enhance aggregation of a polyQ-containing protein, probably via activating MEKK1. Unfortunately, technically it was not feasible to directly test the possibility that MEKK1 is essential

## CFTR



## Luc



**Figure 10.** MEKK1 stimulates aggregation of proteins lacking polyQ domain. Confocal microscopy. Top, HeLa cells were transfected with GFP-tagged CFTR either alone (Control or MG132) or along with active MEKK1 mutant (MEKK1). After transfection one sample transfected with CFTR alone was incubated with 5  $\mu$ M of proteasome inhibitor MG132 for 15 h (MG132). Bottom, HeLa cells were transfected with GFP-tagged luciferase either along with active MEKK1 mutant (MEKK1) or alone (all other pictures). Cells transfected with luciferase alone after transfection were either left untreated (Control [living cells], and Dead cell) or heated at 44°C for 30 min followed by 15-h recovery (Heat Shock).

for this stimulation of aggregation, because the kinase-dead mutant of this kinase did not manifest a sufficient inhibitory effect. The observed potentiation of IB formation by nocodazole and cytochalasin B (Fig. 8) may also result from activation of stress signaling by these agents.

There are several possible reasons why a mechanism for regulation of protein aggregation by a stress-activated signaling pathway has evolved. Abnormal proteins that

emerge in the cell due to mutations, chemical modifications, or stress-induced denaturation may either be refolded by chaperones or degraded via the ubiquitin-proteasome pathway. If these processes are not efficient enough and abnormal proteins accumulate, they become toxic. Under these conditions forced protein aggregation leading to IB formation may become an efficient mechanism to rapidly reduce the levels of the unfolded molecules in the soluble phase, thus representing a cellular defense mechanism to segregate and limit the toxic consequences of the abnormal proteins. On the other hand, if IBs are involved in initiation of an apoptotic program, the regulated formation of IBs may serve to eliminate potentially damaged cells. Obviously, accumulation of abnormal polypeptides is most likely to occur under stressful conditions, and therefore it is reasonable that this process is upregulated by a stress-activated protein kinase MEKK1. The stress-regulated formation of IBs may be especially relevant to neurons in disease. As mentioned in the Introduction, such cells may experience an internal stress and stimulation of stress-activated signaling pathways. Therefore, MEKK1-mediated stimulation of formation of IBs may be an adaptive mechanism designed to reduce neurodegeneration by protecting from apoptosis, or eliminating defective neurons.

As reported previously, in contrast to other members of this protein kinase family, MEKK1 associates with discrete spotted cellular structures (Christerson et al., 1999). We confirmed this observation (Fig. 7 A) and found that a large fraction of the overexpressed MEKK1 mutant associates with the 400 g pellets while retaining its activity. In contrast, a much lower fraction of inactive MEKK1 mutant associated with this fraction (Fig. 5 A). Furthermore, when coexpressed, 104QP IB colocalized with one or several of MEKK1-containing structures. These data suggest that MEKK1 may play a direct role in formation of an IB. We could not detect direct association of MEKK1 with soluble 104QP by coimmunoprecipitation (not shown). We were also unable to observe any change of the size of soluble 104QP complex in response to expression of active MEKK1. These data suggest that MEKK1 $\Delta$  does not directly interact with soluble 104QP. Rather, as discussed below, it seems to activate the nucleation process. One of the possibilities is that MEKK1 associates with a component of the cytoskeleton and facilitates an IB seeding event by phosphorylation of a factor, which triggers the IB nucleation. We were unable to observe involvement of known MEKK1-activated pathways in aggregation of polyQ. Also, we found that the activated form of Raf, another kinase that stimulates MAPK cascades, was not competent in stimulation of 104QP aggregation when coexpressed with this polypeptide. Therefore, it seems either that MEKK1 can directly activate the aggregation process, or that there is a distinct, yet unknown signaling pathway activated by this kinase.

In this work we also investigated basic events in formation of an IB by a polyQ-containing polypeptide. There was an unexpected finding that 104QP did not form multiple homooligomers in the process of aggregation. This molecule was only found in a soluble form in a discrete complex of  $\sim$ 350 kD, or in very large structures precipitated at 400 g, most likely associated with components of

the cytoskeleton. We found that polyQ-containing IBs have certain similarities with aggresomes, but also important differences. Like aggresomes, the IBs localized to the centrosome were surrounded by intermediate filaments, and recruited major chaperones. On the other hand, IB formation was not inhibited by nocodazole or cytochalasin, and therefore did not require either microtubules or actin filaments, at least in HeLa cells. This finding is in striking contrast to aggresome formation, which involves transport of microaggregates along the microtubules.

Based on these findings, we propose that formation of IBs by polyQ polypeptides involves a nucleation step, followed by growth of an IB because of direct association of soluble 104QP complexes with a seed (a "snowball" mechanism). 104QP nucleation appears to be the only rate-limiting step because once an aggregation seed emerges, all the polypeptides in a cell aggregate within <20 min (Kazantsev, A., unpublished). Hence, MEKK1 is likely to facilitate only the nucleation of polyQ. Because large IBs are found associated with the centrosome, it is likely that MEKK1-initiated nucleation sites are preferably formed within the centrosome. On the other hand, multiple IBs are seen in a fraction of cells. Therefore, it appears that several minor nucleation sites could also be formed at other locations in the cytosol as well. Even if nucleation is initiated at multiple sites in a cell, 104QP may still form a single IB because once the first nucleation site emerges, all the soluble 104QP will rapidly aggregate, and the other nucleation sites would not play a role. However, they may become important for the less aggregation-prone 47Q. For this polypeptide, whereas the nucleation step is still rate limiting, the growth of an IB is probably a slow and reversible process. Slow aggregation of 47Q at multiple sites may lead to its inability to form an IB large enough to become visible under the microscope. Interestingly, when growth of 104QP-containing IB is artificially inhibited, one can observe appearance of multiple small "seeds" in a cell (Kazantsev, A., unpublished). It is noteworthy that minor nucleation sites may become especially important in very extended neuronal cells. In fact, in such cells visible aggregates were observed not only in the cell body where the centrosome is located, but also in the axon (Li et al., 1999). It is possible that in neurons the preformed aggregates may be transported to a centrosome via microtubules.

The effects of MEKK1 on protein aggregation are not limited to the polyQ-containing proteins. Indeed, MEKK1 was able to facilitate aggregation of CFTR and luciferase. CFTR was the first polypeptide to be shown to form an aggresome (Johnston et al., 1998). In HeLa cells we did not see association of CFTR with a single spot-like body, but rather with the crescent-like structure in close proximity to a nucleus (Fig. 10), suggesting that the appearance of aggresomes may vary depending on cell type and other factors. Activation of CFTR aggregation by MEKK1, as well as by proteasome inhibitors, was associated with accumulation of this polypeptide. The effect of the proteasome inhibitors was attributed to stabilization of a short-lived CFTR, leading to formation of IBs. It is unlikely that MEKK1 also stimulates CFTR aggregation through regulation of its degradation. Rather, MEKK1 enhances aggregation, which may render CFTR less susceptible to the degradation machinery, resulting in accumulation of this

polypeptide in IB. Although these alternatives cannot be dissected experimentally, the fact that MEKK1 was able to facilitate aggregation of relatively stable (not shown) polyQ polypeptides, is in line with the latter scenario. It is noteworthy that proteasome inhibitors activate stress signaling pathways (Meriin et al., 1998), including MEKK1 (Meriin, A.B., unpublished data). Therefore, MEKK1 activity may contribute to the stimulation of protein aggregation by proteasome inhibitors.

The most intriguing finding is how MEKK1 enhances aggregation of luciferase, which does not aggregate spontaneously. The fact that luciferase aggregates when cells are exposed to mild heat shock suggests that the luciferase conformation can easily be destabilized. There is probably a constant process of unfolding and refolding of luciferase at normal temperature but because of the lack of nucleation no aggregates are formed. MEKK1 may facilitate creation of nucleation sites, which would lead to capturing unfolded molecules at these sites and to shifting the equilibrium towards aggregation.

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