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ARTICLES

Modulation of the ERK Pathway of Signal Transduction by Cysteine Proteinase Inhibitors

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Abstract Cell proliferation requires the coordinate synthesis and degradation of many proteins. In addition to the well-characterized involvement of the proteasome in the degradation of several cell cycle-regulated proteins, it has been established that cysteine proteinases are also involved in the control of cell proliferation, but their role is currently not understood. By using both synthetic cysteine proteinase inhibitors and overexpression of T-kininogen (T-KG), a physiologically relevant cysteine proteinase inhibitor, we show that inhibition of cysteine proteinases results in a severe inhibition of the ERK pathway of signal transduction. Mechanistically, this effect appears to be the result of stabilization of the ERK phosphatase MKP-1, which leads to an enhanced dephosphorylation (and hence inactivation) of ERK molecules. These results are specific to cysteine proteinase inhibitors and are not observed when either serine proteinases or the proteasome are inhibited. We hypothesize that inhibition of cysteine proteinases in vivo leads to a dysregulation of the ERK pathway, which results in an inability of the cell to transmit to the nucleus the signals generated by the presence of growth factors, thus resulting in loss of cell proliferation. J. Cell. Biochem. 80:11–23, 2000.

Key words: MAP kinase; proteolysis; T-kininogen; cell proliferation; aging

Progression of cells through the cell cycle requires the activity of several cyclin dependent kinases, CDKs. While the abundance of CDK molecules does not show significant changes throughout the cell cycle, their activity is directly modulated by their association with both positive (cyclins) and negative (CDK inhibitors, CKIs) effectors. Cell cycle progression requires the orderly synthesis and degradation of these effectors [Atherton-Fessler et al., 1993; Coats et al., 1996], and the degradation of several cyclins and CDKs has been shown to be dependent on proteasome activity [Chun et al., 1996; Murray et al., 1998; Tanaka and Chiba, 1998]. In contrast and in spite of several reports [March et al., 1993; Mellgren, 1997; Shoji-Kasai et al., 1988], the role of cysteine proteinases in the control of cell proliferation is less clear. By using synthetic aldehyde inhibitors of cysteine proteinases, March et al. [1993] have shown that progression of vascular smooth muscle cells through the cell cycle requires at least three different steps in which cysteine proteinases are involved. Our own data indicates that expression of the cysteine proteinase inhibitor T-kininogen results in inhibition of cell proliferation, primarily at a step located near the end of G_1, and possibly coinciding with the restriction point. Recent research indicates that calpains are the cysteine proteinases most likely involved in arresting cell cycle progression late in G_1 [Mellgren, 1997; Mellgren et al., 1994].

In addition to cell cycle proteins such as those described above, cell proliferation in vitro requires the sensing of growth factors present in the culture medium and the transmission of this information to the nucleus via signal transduction processes. By far, the most studied signal transduction cascade involved in the

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control of cell proliferation is the extracellular signal regulated kinase (ERK) pathway [Boulton and Cobb, 1991]. Activation of this pathway leads to the phosphorylation of several targets, including other protein kinases [Sturgill et al., 1988], membrane receptors [Northwood et al., 1991], transcription factors [Hill and Treisman, 1995], and cytoskeletal proteins [Reszka et al., 1997]. This in turn results in alterations of such cellular functions as proliferation and differentiation. Indeed, while the proliferative response of fibroblasts clearly requires the activation of MAP (mitogen-activated protein) kinases [Cowley et al., 1994; Lavoie et al., 1996; Meloche et al., 1992; Pages et al., 1993], it has been shown that, depending on the level and duration of ERK activation, some cells respond by either proliferation or differentiation [Marshall, 1995].

Maximal ERK enzymatic activity is associated with dual phosphorylation of the enzyme at both Thr and Tyr residues [Ahn et al., 1991; Anderson et al., 1990; Hunter, 1995]. This mode of activation implies the existence of negative control mechanisms that operate through phosphatases [reviewed in Hunter, 1995; Keyse, 1995; Nebreda, 1994]. Several protein phosphatases can dephosphorylate ERKs in vitro, including several PTPases (protein phosphotyrosine phosphatases [Zhao et al., 1996], as well as the Ser/Thr phosphatase PP2A [Alessi et al., 1995; Braconi Quintaje et al., 1996]. On the other hand, a likely physiological effector is represented by phosphatases of the MKP (MAP kinase phosphatase) family, which are dual phosphatases that specifically dephosphorylate both Thr and Tyr in MAP kinases such as ERK1 and ERK2 in a variety of cell types [Duff et al., 1995; Krautwald et al., 1995; Sun et al., 1993].

In this study, we wished to further elucidate the role of cysteine proteinases in cell proliferation. In addition to the use of classical synthetic cell permeable inhibitors of this class of enzymes, we also used cell lines that express T-KG under the control of the mouse metallothionein promoter (MT-1). The choice of this particular cysteine proteinase inhibitor is based on our previous observations that indicate that T-KG expression is considerably increased in the liver of old rats [Sierra, 1995; Sierra et al., 1992; Sierra et al., 1989]. T-KG is a multifunctional protein whose best characterized functions are as a cysteine proteinase inhibitor [Sueyoshi et al., 1985] and as a precursor to the vasoactive peptide T-kinin [Berg et al., 1991]. We have shown that both in the liver during aging [Keppler et al., 1997] and in vitro in our cell cultures, (Felipe Sierra, unpublished observations), expression of the protein leads to intracellular accumulation of biologically active T-KG, capable of inhibiting cysteine proteinases such as cathepsins B and L.

As a model to study both the role of T-KG overexpression in the aged animal, and the potential role of this specific cysteine proteinase inhibitor in signal transduction, we have treated Balb/c 3T3 cells with several synthetic proteinase inhibitors and measured the effect on both the activity and steady state levels of proteins involved in the ERK pathway. Similarly, we have tested the same parameters in fibroblast cell lines that express biologically active T-KG. Our results indicate that T-KG expression results in inhibition of cell proliferation and arrest in late G_1. Here, we show that inhibition of cysteine proteinases, either by synthetic inhibitors or by expression of T-KG, results in a significant reduction in the basal level of ERK enzymatic activity. Paradoxically, this is accompanied by a marked increase in the steady state level of both ERK1 and ERK2. These observations are explained by a mechanism whereby T-KG stabilizes both ERKs and the phosphatase MKP-1 against proteolytic degradation, thus resulting in the observed decrease in ERK enzymatic activity. Thus, we postulate that cysteine proteinase inhibitors such as T-KG can play an important role in regulating the activity of the ERK MAP kinase pathway of signal transduction in vivo.

MATERIALS AND METHODS

Reagents

Media and serum were purchased from Life Technologies Inc. All antibodies were from Transduction Laboratories, except the antibody to MKP1 (Santa Cruz) and the antibody against β-actin, which was purchased from ICN. Other chemicals were from Sigma, unless specified.

Cell Culture

Cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin sulfate

(100 μg/ml). Cells were grown in a humidified 5% CO_2 atmosphere at 37°C. They were plated at 1×10^4 cells/cm^2 and allowed to grow for 24 h. When necessary, protease inhibitors were added 24 h after plating. Unless otherwise indicated, cells were collected for analysis 48 h after plating.

Stable Transfection

The isolation and characterization of full-length T-KG cDNA clones [Sierra et al., 1989] as well as the preparation of constructs containing this cDNA under the control of the mouse MT-1 promoter have been described elsewhere. Mouse fibroblasts (L_{TK}- and Balb/c 3T3) were transfected by standard procedures, and transfected cell lines were selected by growth in 400 μg/ml G418. In this report, we have further analyzed L_{MK6} cells, which are based on an L_{TK}- background, and both $B_{2.3}$ cells (whose vector alone control are B_{MCN-4} cells), and $B_{K.4}$ cells (whose control are B_{PUC-2} cells), both of which are based on a Balb/c 3T3 background. It should be noted that, even though we used an inducible promoter, expression of T-KG in all our cell lines is very leaky and only moderately affected by the presence of heavy metals. Therefore, all experiments were performed in the presence of heavy metal induction.

Immunoblotting

Total cellular extracts were prepared by washing the cell monolayers twice in ice-cold PBS, followed by lysis for 30 min at 4°C with rocking in a modified Hibi buffer [Hibi et al., 1993] consisting of 25 mM Hepes, (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 2 mM Na pyrophosphate, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonyl fluoride. Particulate matter was removed by centrifugation at 4°C, and protein concentration was determined by the Bradford method as commercialized by BioRad. A 30–50 μg aliquot of each sample was separated by 10% SDS-PAGE and electrotransferred to nitrocellulose. Western blotting was performed under standard conditions using 5% nonfat milk as a blocking agent, horseradish peroxidase-conjugated goat antirabbit or antimouse IgG (Transduction Laboratories) as secondary antibodies, and the ECL system (Boehringer Mannheim) for detection. In all cases, data from Western blot analysis was normalized by stripping and reprobing the membranes with an anti-β actin monoclonal antibody (ICN).

MAP Kinase Activity Assays

MAP kinase (ERK) activity was determined using a commercially available MAP kinase assay kit (Life Sciences). The kit supplies a peptide derived from the EGF receptor as a specific substrate for ERK activity that can be phosphorylated by cellular extracts in the presence of [γ-^{32}P]ATP. Activity is quantitated by TCA precipitation on phosphocellulose filters, followed by scintillation counting. Assays were performed in triplicate as per the manufacturer.

In some cases, ERK activity was determined by Western blotting using anti-active MAP kinase antibodies (New England BioLabs). For these experiments, Western blot analysis was performed exactly as suggested by the manufacturer, and enzyme activity was expressed as a ratio between the signals obtained with this antibody and that obtained with anti-β actin antibodies (ICN).

Phosphatase Activity Assays

To measure ERK-specific phosphatase activity, 50 μg of the synthetic peptide ADPDHDHTGFLTEYVATRWRR (derived from human ERK1, Santa Cruz Laboratories), were labeled in vitro with [γ-^{32}P]ATP in the presence of MEK that was immunoprecipitated from Balb/c 3T3 cells stimulated to leave quiescence by a 15 min pulse with 20% serum. The labeling reaction was performed for 12 h in 200 μl of MEK buffer (25 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT) at 30°C. The labeled peptide was then dialyzed for 35 h at 4°C against an excess of 25 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35. MAP kinase phosphatase activity was determined by incubating 20 μl of the resulting ^{32}P-labeled peptide (in a final volume of 60 μl) with different amounts of total proteins extracted from logarithmically growing B_{MCN4} or $B_{2.3}$ cells for 15 min at 30°C in phosphatase buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 0.01% Brij 35, 1 mg/ml BSA). The reaction was stopped by addition of 10 μl orthophosphoric

acid, and the radioactivity still associated to macromolecules was determined by applying 50 μl of the sample to phosphocellulose filters, followed by extensive washes in 75 mM phosphoric acid, 1% acetic acid.

Total tyrosine phosphatase activity was determined by a method based on the measurement of ^{32}P liberated from Myelin Basic Protein that had been labeled in multiple tyrosine residues by the action of Abl tyrosine kinase (20 U/μl) in the presence of [γ-^{32}P]ATP. Both the labeling and the tyrosine phosphatase assays were done using a kit from New England BioLabs, and were performed essentially as described by the supplier.

Degradation Rates

Cells were seeded as before. After 48 h in culture, cycloheximide was added to a concentration of 10 μg/ml, and incubation was continued for the specified times. Total cellular extracts were prepared as described above, and 50 μg of proteins were analyzed by Western blot analysis. Experiments were done at least in duplicate in each cell line, and in all cases, data were normalized to the signal observed in the corresponding cells in the absence of cycloheximide.

RNA Preparation and Analysis

Total RNA was prepared essentially by the AGPC method [Chomczynski and Sacchi, 1987], with some modifications [Puissant and Houdebine, 1990]. Total RNA (5 μg) was electrophoretically fractionated on glyoxal gels, transferred to GeneScreen membranes (Dupont, NEN Research Products, Boston, Mass.), fixed by exposure to UV light and hybridized to random-labeled partial cDNA probes. The probe for ERK1 was a kind gift of Dr. M. H. Cobb (University of Texas Southwestern Medical Center) and the probe for MKP1 was prepared by RT-PCR from Balb/c 3T3 cells, using the following oligonucleotides:

5′-GGGAATTCCACAACAATGACTTGACCGC-3′
5′-GGAAGCTTAGCTACAAACCTACACTGGC-3′

After PCR, the amplified band of the expected size was excised from an agarose gel, purified, and labeled by random priming. After hybridization and stringent washing, membranes were exposed wet to X-ray film.

Data Analysis

Films were scanned and quantification was performed on the resulting autoradiograms using BioQuant Image Analysis System software (Molecular Dynamics). With the exception of the degradation rate assays, all Western blot data were normalized against β-actin as a control for gel loading. All experiments were performed at least three times, with similar results, and analyzed using a two-tailed, paired Student's t-test. Values were considered to be significantly different if P was less than 0.01.

RESULTS

Cysteine Proteinase Inhibitors Down-Regulate the ERK Pathway

Logarithmically growing cultures of Balb/c 3T3 cells were treated with several synthetic protease inhibitors for 6 h. Figure 1A shows that inhibition of cysteine proteinases by either E64D or calpeptin leads to a dramatic inhibition of ERK activity. In contrast, inhibition of serine proteases by TLCK or inhibition of the proteasome by LLnL does not result in a measurable change in ERK activity. To examine the mechanism responsible for this decreased ERK activity, we measured the steady state level of the enzymes, ERK1 and ERK2. Figure 1B shows that the steady state level of ERK1 is slightly but reproducibly elevated in cells treated with cysteine proteinase inhibitors. In contrast, TLCK and LLnL have no effect on this parameter. A similar result was obtained with ERK2 (data not shown). Thus, we observe that inhibition of cysteine proteinase activities leads to a dysregulation of the ERK pathway, characterized by a dichotomy between the steady state level of ERK proteins (increase) and their enzymatic activity (decrease).

ERK Activity Is Decreased in the Presence of T-KG

In an attempt to determine whether a physiologically relevant cysteine proteinase inhibitor could also affect the activity and steady state level of ERK proteins, we used total cellular extracts from our T-KG-expressing fibroblast-derived cell lines to measure MAP kinase-dependent (ERK1 and ERK2) phosphorylation of an EGF receptor peptide. The results, shown in Figure 2, indicate that the activity of these enzymes (ERK1 plus ERK2) is reduced 4-fold ($P < 0.001$, $N = 3$) in $B_{2.3}$ cells,

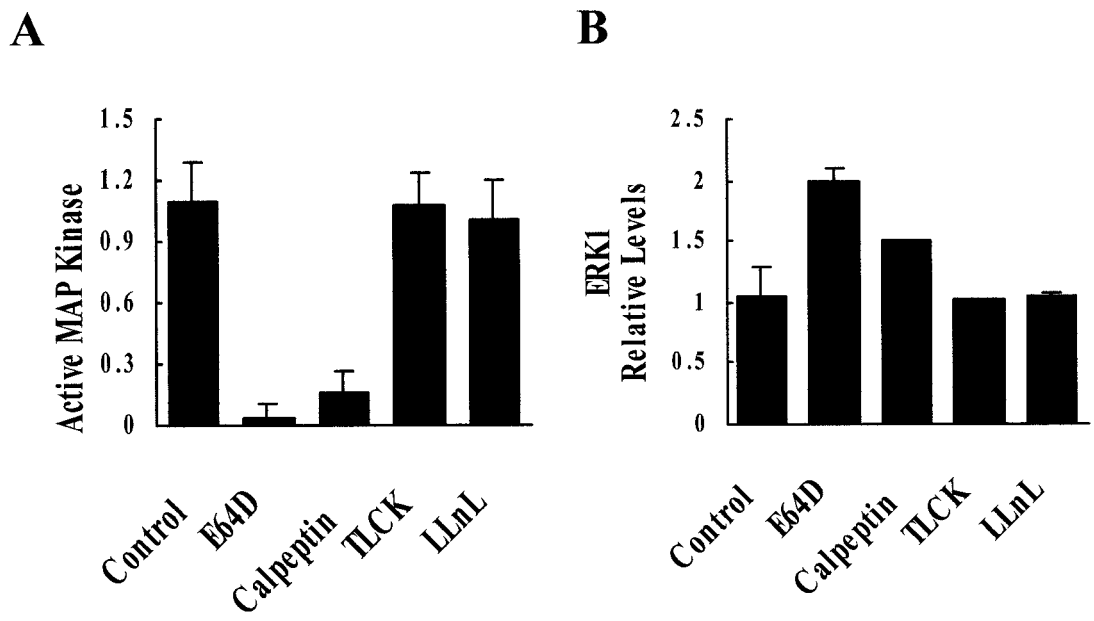

Fig. 1. Effect of synthetic protease inhibitors on the ERK pathway. (**A**): Inhibition of MAP kinase activity. Logarithmically growing Balb/c 3T3 cells were treated for 6 h with vehicle alone (Control) or with 50 μM E64D, 50 μM calpeptin, 50 μM TLCK, or 100 μM LLnL. Total cellular extracts were prepared, and a 50 μg aliquot was separated by 10% SDS-PAGE and electrotransferred to nitrocellulose. The membranes were probed with anti-β-actin (ICN) and, after stripping, with anti-active MAP kinase antibodies (New England BioLabs) under the conditions specified by the supplier. The data represents the average of three independent measurements, and it is plotted as a ratio of active MAP kinase over β-actin. The signal obtained at time zero was given a value of 1, and Control represents the signal obtained in the absence of any inhibitor. (**B**): Steady state level of ERK1. The same membrane was used to probe for the steady state levels of ERK1 and ERK2. Again, data is presented relative to β-actin, and the signal obtained in the absence of inhibitors was given a value of 1.

as compared to their vector-alone transfected counterparts. Similar results have been obtained with the other cell lines (data not shown).

In Spite of the Decrease in ERK Activity, the Steady State Level of ERK Proteins Is Specifically Increased in T-KG-Expressing Cells

Since we found that inhibition of cysteine proteinases leads to a slight but reproducible increase in the steady state level of ERK proteins (Fig. 1B), we have also determined the effect of T-KG expression on the steady state level of ERK proteins. Figure 3A shows that T-KG expression leads to a significant and reproducible increase in the steady state level of both ERK1 and ERK2 in all three cell lines tested. In contrast, T-KG has little or no effect on the steady state level of Ras, Raf-1, PKCα, MEK1, JNK-1, actin or STAT 3 (data not shown). Thus, these results suggest that T-KG overexpression leads to an increase in the steady state level of both ERK isoforms, and

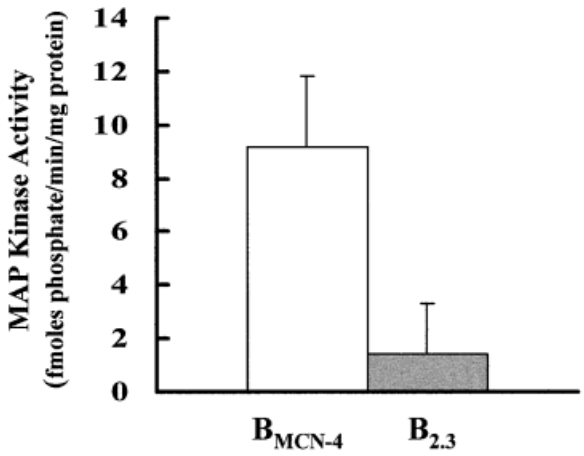

Fig. 2. MAP kinase activity is inhibited by expression of T-KG in fibroblast cell lines. An aliquot (10 μl) of total cellular extracts from a T-KG expressing cell line ($B_{2.3}$) and its vector-alone control (B_{MCN-4}) was used to measure MAP kinase (ERK) activity in solution, using a commercial kit from Amersham. The assay measures phosphorylation of a specific peptide derived from the EGF receptor, in the presence of [γ-^{32}P]ATP. Activity was quantitated by TCA precipitation on phosphocellulose filters. Assays were done in triplicate, and the experiment was repeated three times.

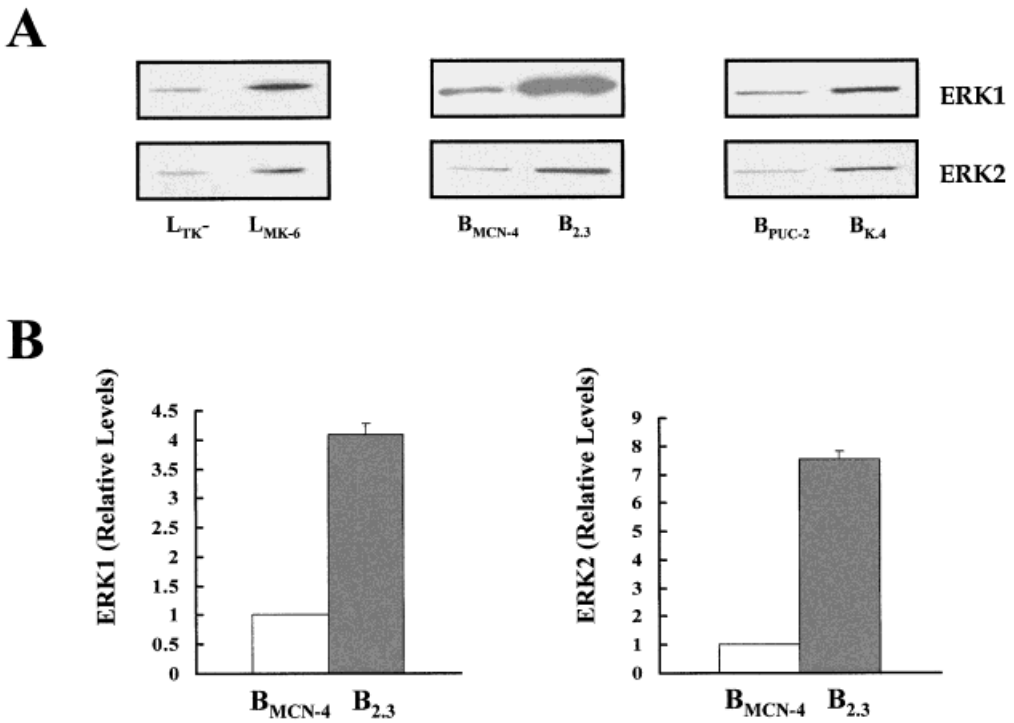

Fig. 3. T-KG expression results in an elevation in the steady state level of ERK proteins. Logarithmically growing cells were collected, lysed, and 50 μg of total cellular proteins were analyzed by Western blot. (**A**): Representative Western blots. The steady state level of ERK1 (top panels) and ERK2 (bottom panels) in three transfected cell lines and their respective controls is shown. (**B**): Quantitation of ERK1 and ERK2 levels. ERK1 (left), and ERK2 (right) relative levels in $B_{2.3}$ and B_{MCN4} cells (N = 7). Each blot was normalized for loading by probing for β-actin steady state levels. Values are given relative to the level found in B_{MCN4} cells, which was assigned a value of 1.

these effects are specific, since other proteins involved in signal transduction by the same (Ras, Raf-1, MEK1, PKCα) or other (JNK-1, STAT 3) pathways are not similarly affected. Furthermore, our results suggest that ERK might be normally degraded by cysteine proteinases, and that inhibition of these enzymes by T-KG could result in severe imbalances in the Ras/MAP kinase pathway of signal transduction. Figure 3B shows the quantitation of seven independent experiments. This quantitation of the Western blot analyses indicates a highly reproducible ($P < 0.001$) 4-fold (ERK1) or 8-fold (ERK2) increase in the steady state level of ERK proteins in T-KG-producing cell lines.

Thus, as in the case of synthetic cysteine proteinase inhibitors (Fig. 1), our results in T-KG-expressing cell lines indicate a dramatic dichotomy between the increase in the steady state levels of both ERK1 and ERK2 (Fig. 3) and the decrease in their activity (Fig. 2). The experiments shown were performed comparing similar amounts of total cellular proteins in each case and, therefore, assuming that T-KG overexpression does not significantly affect cell volume or mass, the results indicate that the physiological effect of T-KG is a 4-fold reduction in ERK activity per cell. Since this effect happens in the presence of a 4- to 8-fold increase in the steady state level of ERK proteins, the results further suggest that, per unit of immunoreactive ERK, T-KG induces a 16- to 32-fold reduction in MAP kinase specific activity.

The Steady State Level of MKP1 Is Increased Both by Addition of Cysteine Proteinase Inhibitors and in T-KG-Expressing Cells

Our results indicate that inhibition of cysteine proteinases leads to a decrease in ERK specific enzymatic activity. This could be the result of either decreased phosphorylation (by MEK) or increased dephosphorylation (by a phosphatase). Our data indicates that the steady state level of MEK 1 is not affected by

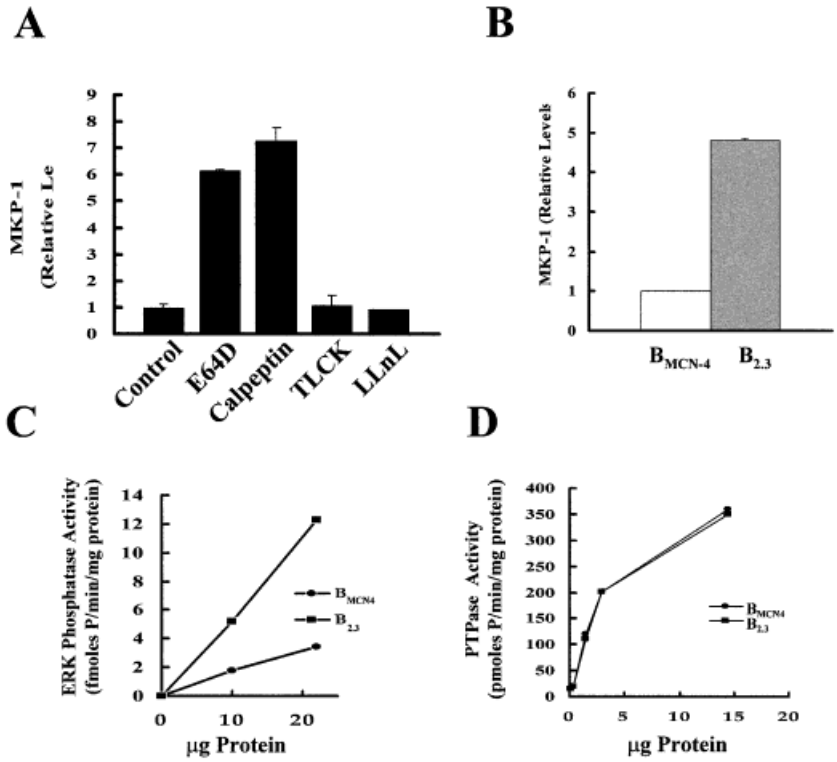

Fig. 4. The steady state level of MKP-1, as well as MAP kinase phosphatase activity are both increased in the presence of cysteine proteinase inhibitors. (**A**): MKP-1 steady state level in the presence of synthetic inhibitors. Samples were prepared as described in Figure 1, and the same membranes were probed for MKP-1. Results are given as a ratio of MKP-1 to β-actin, and the ratio found in control cells was assigned a value of 1. (**B**): MKP-1 steady state level in T-KG expressing cells and their controls. Samples were prepared as described in Figure 3 and analyzed by Western blot, using anti-MKP-1 antibodies. The data represents the quantitation of four independent experiments. (**C**): MAP kinase phosphatase activity in T-KG expressing cells and their controls. ERK-specific phosphatase activity was measured in total cellular extracts using an in vitro phosphorylated ERK peptide, as described in Materials and Methods. (**D**): Total tyrosine phosphatase activity in T-KG expressing cells and their controls. These measurements were done in total cellular extracts, using a kit from New England BioLabs, as specified by the supplier.

the presence of T-KG (data not shown), and therefore we concentrated our analysis on the phosphatases. The phosphatases most likely involved in directly regulating ERK activity are the MKP family members, of which there are about a dozen genes cloned [Tanoue et al., 1999]. The results (Fig. 4A) show that the cysteine proteinase inhibitors E64D and calpeptin, but not inhibitors of other classes of proteases, lead to a significant increase in the steady state level of MKP-1. Similarly, Figure 4B indicates that the steady state level of MKP-1 is also significantly increased in cells that produce T-KG. The increase in the level of MKP1 (5-fold, $P < 0.001$, Fig. 4B) is comparable to the increase in ERK1 steady state level, strongly suggesting the participation of MKP-1 in the observed inhibition of MAP kinase activity in $B_{2.3}$ cells.

Since MKP-1 has been characterized as an early response gene, whose mRNA levels increase dramatically in response to serum stimulation [Misra-Press et al., 1995; Sun et al., 1993; Zheng and Guan, 1993], it is generally assumed that MKP-1 activity follows its steady state levels. On the other hand, a similar assumption is often made with respect to ERK, and our results indicate that such an assumption can be misleading. Therefore, we next measured directly the effect of T-KG on total MAP kinase phosphatase activity. For this, we measured the release of ^{32}P from a human ERK1-derived peptide that was dually phosphorylated in vitro with immunoprecipitated MEK1 obtained from serum stimulated Balb/c 3T3 cells. This approach therefore includes the activity of MKP-1, as well as several other phosphatases known to dephosphorylate ERK

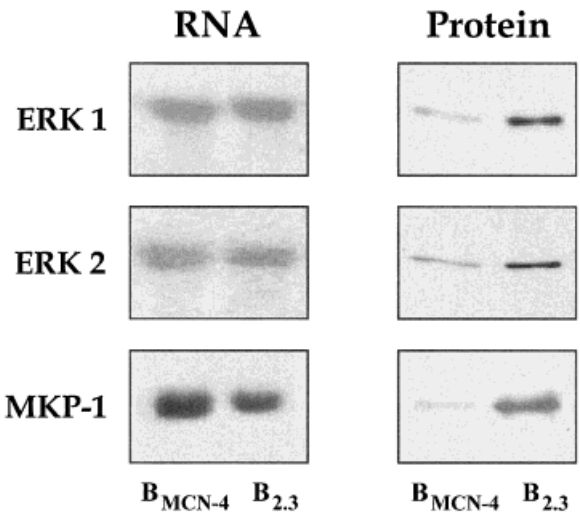

Fig. 5. ERK1, ERK2, and MKP-1 mRNA and protein levels in T-KG expressing cells and their controls. Total RNA (left panels) or total cellular proteins (right panels) from $B_{2.3}$ cells and their respective controls, grown for 24 h, were prepared as described in Materials and Methods. ERK1, ERK2, and MKP-1 mRNA levels (left panels) were analyzed by Northern blot, using 5 μg of total RNA. Protein levels (right panels) were analyzed by Western blot as indicated in Figure 3.

proteins. The results in Figure 4C indicate that T-KG-expressing cells indeed have a 3-fold increase in phosphatase activities capable of dephosphorylating ERK1, as compared to their controls. This effect appears to be specific, as total tyrosine phosphatase activity is not affected by the presence of T-KG (Fig. 4D). The increase in MAP kinase phosphatase activity in cells that overexpress T-KG could play an important role in the decrease in the basal level of ERK activity observed in these cells.

The Rate of Degradation of ERK1 and MKP1 Is Specifically Decreased in T-KG-Expressing Cells

The increase in the steady state level of these two proteins could be due to increased expression or decreased degradation. We have used Northern blot analysis to determine the mRNA level of both ERK1, ERK2, and MKP1 in $B_{2.3}$ and control B_{MCN-4} cells. Figure 5 shows that although the steady state level of these proteins is elevated in T-KG-producing cell lines, there is little or no difference in steady state mRNA levels. This suggests that the increase in protein levels is not controlled at the level of mRNA abundance, and thus, T-KG is likely to affect both ERK and MKP-1 stability, probably by a postranscriptional mechanism involving inhibition of the relevant proteases. We thus tested directly whether T-KG can affect the degradation rate of ERK or MKP proteins. For this, we measured the disappearance of the proteins in question during a pulse of cycloheximide treatment (10 μg/ml, a concentration shown to inhibit de novo protein synthesis). Under these conditions, Western blot analysis indicates the rate of degradation of immunoreactive proteins as a function of time. Figures 6 and 7 show the results of this experiment for several proteins, in both a T-KG-expressing cell line and its control. The results indicate that T-KG leads to a significant stabilization of both ERK1 and MKP1 proteins. In contrast, the degradation rate of STAT 3 is not significantly affected by the presence of T-KG. The effect is most pronounced for those proteins (ERK1 and MKP1) whose apparent half-lives in the parental cells is the shortest (15–20 min for ERK1, 40–50 min for MKP1). In the case of ERK2 (apparent half-life in the control cell line is 90 min), the effect of T-KG appears less dramatic but still statistically significant (data not shown). We observed no statistically significant difference in the rate of disappearance of STAT 3 (apparent half-life is 60 min). All experiments have been repeated at least twice. Figure 7 represents a similar set of data, but expressed as percentage of the initial signal remaining after 2 h of exposure to cycloheximide. These results further confirm the kinetic data presented in Figure 6, in that T-KG-producing cells display a significant protection against proteolytic degradation of both ERK1, ERK2, and MKP1, while failing to protect STAT 3 against proteolytic degradation. It should be noted that these data show a complete absence of degradation of ERK1, ERK2, or MKP1 in T-KG-expressing cells, even after 2 h of exposure to the drug.

DISCUSSION

Our results indicate that both treatment of cells with synthetic cysteine proteinase inhibitors and overexpression of a specific cysteine proteinase inhibitor, T-KG, leads to a dramatic dysregulation of the Ras/MEK/ERK pathway of signal transduction. This dysregulation is characterized by a specific increase in the steady state levels of ERK1, ERK2, and MKP-1, accompanied by a parallel decrease in total ERK activity. Indeed our results indicate that, per cell, T-KG leads to a 4-fold reduction

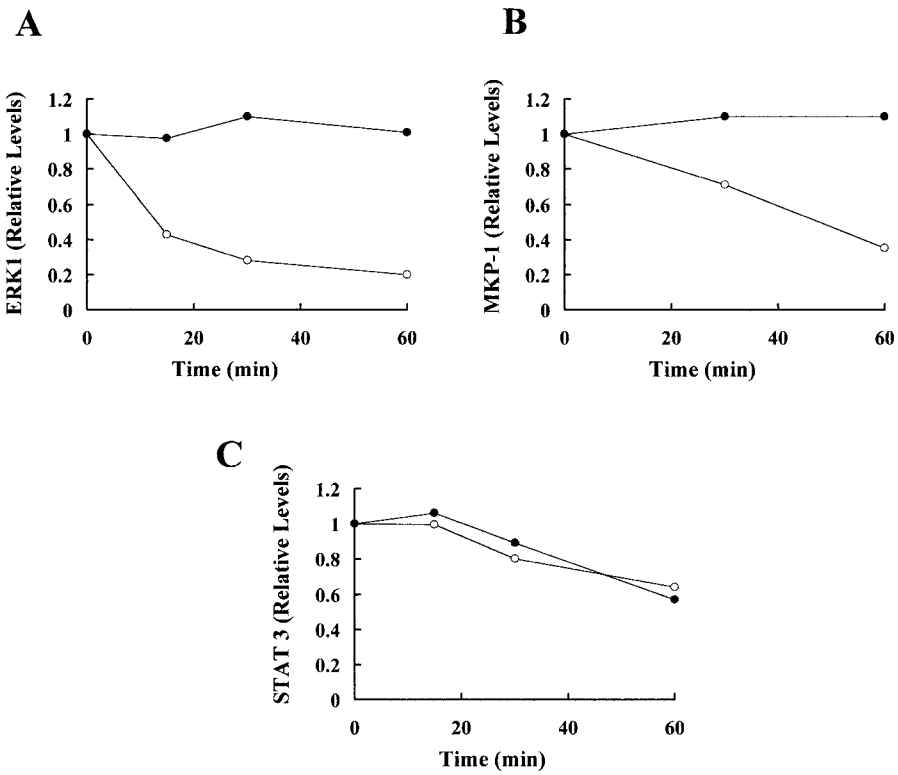

Fig. 6. Effect of T-KG on the degradation rates of ERK1, MKP-1, and STAT 3. Logarithmically growing cells were incubated in the presence of cycloheximide (10 μg/ml) for the times shown. Total cellular extracts were prepared, and 50 μg of protein were analyzed by Western blot. **Panel A:** ERK1; **Panel B:** MKP-1; **Panel C:** STAT 3. Open circles: Vector alone transfectants. Closed circles: T-KG expressing cells. Values are given relative to time zero, and in this case, no correction for loading was done, since β-actin is also slowly degraded during the course of the experiment. The experiment was done in duplicate and in two different cell lines.

in ERK activity, even though each cell has 4- to 8-fold more enzyme in terms of amount. In other words, taken together, these results indicate a 16- to 32-fold reduction in ERK specific activity in T-KG-producing cells, defined as enzymatic activity per unit of immunoreactive protein. Qualitatively and statistically similar results have been obtained in two different T-KG-expressing cell lines analyzed. At the cellular level, our results indicate that T-KG overexpression should lead to a decreased responsiveness to extracellular signals, as well as decreased cell proliferation.

The changes in steady state level of ERK1, ERK2, and MKP-1 are best explained by a posttranscriptional mechanism, since their respective mRNA levels do not increase in the presence of T-KG. Furthermore, our results indicate that T-KG expression indeed results in the specific stabilization of ERK1, ERK2, and MKP-1 proteins in the presence of the protease

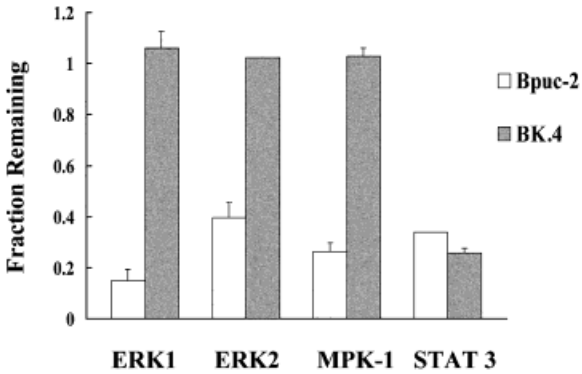

Fig. 7. Fraction of ERK1, ERK2, MKP-1, and STAT 3 remaining after 2 h of cycloheximide treatment. Logarithmically growing cells were incubated in the presence of cycloheximide (10 μg/ml) for an additional 2 h. Total cellular extracts were prepared, and 50 μg of protein were analyzed by Western blot as indicated in Figure 6. The data represents the fraction of each protein remaining at the end of the experiment, relative to the level found at time zero (before addition of cycloheximide). Data was quantitated from the results of three independent experiments.

inhibitor. While alternative scenarios are still possible, our results support the notion that T-KG exerts its action via inhibition of cysteine proteinases. This interpretation is further supported by the fact that addition of the synthetic cell-penetrating cysteine protease inhibitors E64D and calpeptin to Balb/c 3T3 cells also results in similar imbalances in ERK levels and activity. Therefore we postulate that the effects of T-KG on the ERK pathway are mediated by its cysteine proteinase inhibitory activity and that in vivo, these proteins are likely to be degraded by processes directly or indirectly requiring cysteine proteases.

While the rapid responsiveness of the ERK pathway to the extracellular milieu is generally believed to occur primarily via phosphorylation/dephosphorylation [Karin and Hunter, 1995], our results indicate that changes in the intracellular concentration of these enzymes could play an important role in long-term modulation of these pathways. Evidence that changes in the steady state level of relevant proteins can affect the activity of this pathway includes data showing that overexpression of ERK proteins in vitro leads to both an increase in specific kinase activity [Frost et al., 1994], as well as in protein kinase-dependent biological effects [Park and Levitt, 1993]. Similarly, overexpression of a constitutively active form of MEK1 results in a dramatic increase in the expression of cyclin D1, a gene whose activity is controlled via ERK [Lavoie et al., 1996]. Conversely, overexpression of mouse MKP-1 results in a strong inhibition of fibroblast proliferation [Lai et al., 1996; Noguchi et al., 1993; Sun et al., 1994]. In summary, it can be concluded that the overaccumulation of protein kinases results in increased kinase activity, and conversely, overaccumulation of phosphatases results in inhibition of the corresponding pathways. While the examples given above result from direct experimental manipulation of the levels of kinases and phosphatases, it has also been reported that some renal cell carcinomas are associated with increased levels of MEK, which results in increased MAP kinase activity [Oka et al., 1995]. Our experiments further show that changes in the steady state levels of these proteins by physiological means, that is, their impaired degradation, also result in dramatic changes in the activity of the corresponding pathways.

To explain our results, we currently favor a model in which T-KG, acting as a cysteine proteinase inhibitor, inhibits the degradation of ERK1, ERK2, and MKP-1 (Fig. 8). This results in an increase in the cellular levels of these proteins. The increase in MKP-1 leads to the observed increase in dephosphorylated, inactive ERK1/ERK2, resulting in the diminished level of total MAP kinase activity. Since MKP-1 appears to be primarily a nuclear enzyme [Brondello et al., 1995], it is a likely candidate for involvement in the inactivation of nuclearly localized MAP kinases. This model could thus explain the mechanism by which cysteine proteinase inhibitors have a negative effect on cell proliferation [March et al., 1993; Shoji-Kasai et al., 1988].

The effects we describe appear to be specific and do not represent a general imbalance in cell metabolism. This is supported by the following facts: (1) Measurement of protein degradation rates, using pulse/chase experiments followed by total TCA precipitation, indicates that T-KG has little or no effect on the total rate of cellular proteolysis (Felipe Sierra, unpublished observations). (2) The steady state level of several other proteins involved in the Ras/MEK/ERK pathway are not affected by the presence of T-KG. (3) The steady state levels (and in the case of STAT 3, its degradation rate) of proteins involved in other signal transduction pathways, including STAT 3 and JNK, are also not affected by T-KG. (4) The level of phosphotyrosine in total cellular extracts (as measured by quantitation of whole lanes in Western blot experiments) does not change in cells that express T-KG, compared to their corresponding controls (data not shown). Thus, we conclude that it is unlikely that the effects observed represent a general imbalance in the cell, as a result of overt inhibition of proteolytic process.

Our interest in T-KG stems from our observation of a dramatic increase in both its hepatic and serum levels in aging rats [Sierra, 1995; Sierra et al., 1992]. T-KG is an inhibitor of cysteine proteases, and a general decline in the rate of intracellular protein degradation has long been observed to accompany the process of biological aging in a number of phylogenetically diverse organisms [Lavie et al., 1982; Reznick and Gershon, 1979]. Moreover, several groups, including ourselves, have shown that the activity of the ERK pathway is severely

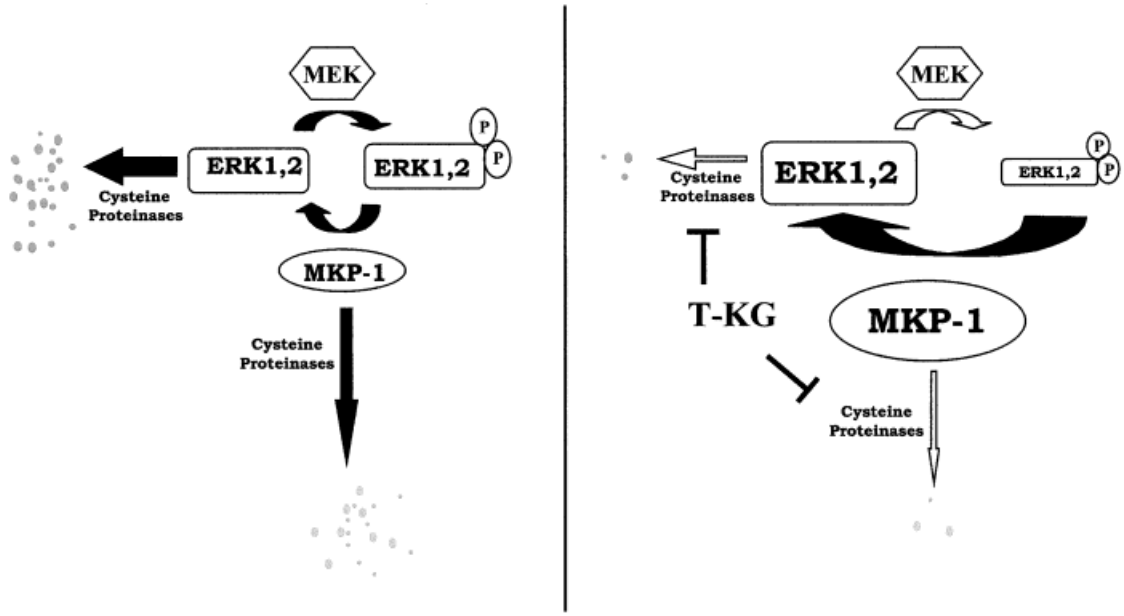

Fig. 8. Model for the effect of T-KG on the activity of the ERK pathway. (**A**): Left diagram. Regulation of ERK1/ERK2 activity in the absence of cysteine proteinase inhibitors. Inactive, dephosphorylated ERK1/ERK2 are phosphorylated by MEK, and dephosphorylated by MKP-1 (or other phosphatases) to their inactive forms. Steady state levels of all relevant proteins is maintained by an equilibrium between their rates of synthesis and degradation. (**B**): Right diagram. Regulation of ERK1/ERK2 activity in the presence of cysteine proteinase inhibitors. ERK1, ERK2, and MKP-1 degradation is impaired in the presence of cysteine proteinase inhibitors, including T-KG. This results in an increase in both ERK1, ERK2, and MKP-1 levels. The increase in MKP-1 level leads to a displacement of the equilibrium toward the dephosphorylated form of ERK1/ERK2, and to inactivation of the ERK pathway.

impaired in cells and tissues derived from old animals. This has been shown both in hepatocytes [Liu et al., 1996], brain (E. Friedman, personal communication), and activated T lymphocytes [Whisler et al., 1996]. In the case of hepatocytes [Liu et al., 1996], as well as the intact liver, it appears that the decreased level of ERK activity correlates with an elevated level of MKP-1. The liver is quantitatively the major site of T-KG production, and the results in this tissue are consistent with our observations in the T-KG overexpressing fibroblast models.

Several reports in the literature have indicated the involvement of proteolysis in the regulation of signal transduction pathways. For example, it has been shown that proteolytic cleavage of PTP 1B [Rock et al., 1997] by calpain leads to activation of the phosphatase activity. Similarly, it has been suggested that calpain activity is also required for down-regulation of PKC by prolonged exposure to phorbol esters [Hong et al., 1995]. Furthermore, a recent report has shown that bromelain, a cysteine proteinase derived from pineapples, can inhibit ERK-2 activation when provided from the outside of the cells [Mynott et al., 1999]. In that study, the authors suggest that the mechanism might be indirect, as the enzyme does not penetrate the cell. To our knowledge, this is the first report on the role of intracellular proteolysis in the control of the ERK pathway. Our previous observation that T-KG gene expression is strongly induced during aging in rats suggests that this mode of regulation might play a significant role in vivo during aging.

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