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Nerve Growth Factor-dependent Survival of CESS B Cell Line Is Mediated by Increased Expression and Decreased Degradation of MAPK Phosphatase 1*

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Paolo Rosini‡, Giovanna De Chiara§, Paolo Bonini‡, Maria Lucibello§¶, Maria Elena Marcocci§, Enrico Garaci§, Federico Cozzolino‡§¶, and Maria Torcia‡‖

From the ‡Department of Clinical Physiopathology, University of Florence, Viale Pieraccini 6, I-50139 Florence, Italy, the §Department of Experimental Medicine, University of Rome "Tor Vergata," Via di Tor Vergata 135, I-00133 Rome, Italy, and the ¶Institute of Neurobiology and Molecular Medicine, National Research Council, Via del Fosso del Cavaliere 100, I-00133 Rome, Italy

The sIgG$^+$ lymphoblastoid B cell line CESS spontaneously produces a high amount of nerve growth factor (NGF) and expresses both high affinity (p140^{Trk-A}) and low affinity (p75^{NTR}) NGF receptors. Autocrine production of NGF maintains the survival of CESS cells through the continuous deactivation of p38 MAPK, an enzyme able to induce Bcl-2 phosphorylation and subsequent cytochrome *c* release and caspase activation. In this paper, we show that NGF induces transcriptional activation and synthesis of MAPK phosphatase 1 (MKP-1), a dual specificity phosphatase that dephosphorylates p38 MAPK, thus preventing Bcl-2 phosphorylation. Furthermore, NGF increases MKP-1 protein stability by preventing its degradation through the proteasome pathway. Following NGF stimulation, MKP-1 protein mainly localizes on mitochondria, suggesting an interaction with p38 MAPK in this compartment. Incubation of CESS cells with *MKP-1*-specific antisense oligonucleotides induces cell death, which was not prevented by exogenous NGF. By contrast, overexpression of native MKP-1, but not of its catalytically impaired form, inhibits apoptosis induced by NGF neutralization in CESS cells. Thus, the molecular mechanisms underlying the survival function of NGF in CESS B cell line predominantly consist in maintaining elevated levels of MKP-1 protein, which controls p38 MAPK activation.

The lymphoblastoid CESS B cell line displays a CD19$^+$, CD20$^-$, CD44$^+$, CD38$^+$, CD77$^-$, and IgGK$^+$ surface phenotype (1), which suggests its origin from an antigen-selected, somatically hypermutated, and proliferating B lymphocyte, a stage ontogenetically close to that of memory B cells (2). Similar to memory B lymphocytes, CESS cells express both high affinity (p140^{Trk-A}) and low affinity (p75^{NTR}) NGF[1] receptors, spontaneously produce high amounts of NGF, and utilize it for their own survival (1, 3). In memory B lymphocytes and in the CESS B cell line, neutralization of endogenous NGF induces activation of p38 MAPK, its mitochondrial translocation, and phosphorylation of Bcl-2 protein (4).

Besides p38 MAPK, several enzymes such as JNK, protein kinase Cα, Cdc2 kinase, CDK6, have been considered responsible for Bcl-2 phosphorylation (5–14) during UV irradiation, exposure to microtubule-targeting drugs, or serum/growth factor deprivation. Bcl-2 phosphorylation, which occurs in serine and threonine residues located in a loop between α1 and α2 helices (11), has been reported both as increasing or decreasing the anti-apoptotic potential of the protein (15). However, in memory B lymphocytes and in CESS cells, p38 MAPK-induced Bcl-2 phosphorylation, which occurs in Ser^{87} and Thr^{56} residues of the loop, (4),[2] causes cytochrome *c* release from mitochondria, caspase activation, and apoptotic death. In contrast, the addition of exogenous NGF induces dephosphorylation of p38 MAPK, which prevents Bcl-2 phosphorylation and cell apoptosis. Continuous deactivation of p38 MAPK and maintenance of the functional and structural properties of Bcl-2 protein appear to be important mechanisms underlying the survival-promoting activity of endogenous NGF.

MAPKs including ERK, JNK, and p38 MAPK are activated by upstream kinases such as MEKs 1 and 2 and mitogen kinase kinases 3, 4, and 6 (16). However, once activated, MAPKs are rapidly inactivated by selected families of protein phosphatases. In particular, dual specificity protein phosphatases play crucial roles in the dephosphorylation/inactivation of MAPKs (17, 18). MAPK phosphatase 1 (MKP-1), also termed CL100 or DUSP1, is a prototypic member of the family of inducible dual specificity phosphatases, dsPTPs (19, 20). It selectively dephosphorylates tyrosine and threonine residues on MAPKs and inactivates them. Although all three MAPKs are potential targets of MKP-1 (21–23), it has been reported that JNK and p38 MAPK are preferentially inactivated by this enzyme (21). Other members of the family of inducible dsPTPs, such as MKP-3 and MKP-4, show higher selectivity to ERK, rather than JNK/SAPK or p38 MAPK, suggesting the presence of an

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‖ To whom correspondence should be addressed: Dept. of Clinical Physiopathology, University of Florence, Viale Pieraccini 6, I-50139 Florence, Italy. Tel.: 39-055-4271368; Fax: 39-055-4271371; E-mail: g.torcia@dfc.unifi.it.

[1] The abbreviations used are: NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; EGFP, enhanced green fluorescent protein; PE, phycoerythrin; PARP, poly(ADP-ribose) polymerase; NTR, neurotrophin receptor; hrNGF, human recombinant NGF; CMV, cytomegalovirus; dsPTPs, dual specificity phosphatases; TBS-T, Tris-buffered saline containing 0.05% Tween 20; PBS, phosphate-buffered saline; SAPK, stress-activated protein kinase.

[2] G. De Chiara, M. E. Marcocci, P. Rosini, M. Torica, and F. Cozzolino, manuscript in preparation.

expanding family of structurally homologous dsPTPs possessing distinct MAPK specificity and subcellular localization as well as diverse patterns of tissue expression (20, 24). Serum and growth factor stimulation, tissue regeneration, oxidative stress and heat shock response, nitrogen starvation, and mitogen stimulation (20, 23, 25, 26) all induce transcription of dsPTPs. In particular, MKP-1 and MKP-2, which are not expressed in quiescent cells, are rapidly induced following serum addition, the proteins being detectable as early as 30 (MKP-1) or 60 min (MKP-2) after stimulation (27).

In this paper, we show that in CESS cells NGF is able to increase gene expression and synthesis of MKP-1, one of the most active p38 MAPK-dephosphorylating enzymes, and inhibits its degradation through the proteasome pathway, thus increasing the concentration of active MKP-1. Furthermore, we show that following NGF stimulation, MKP-1 localizes also on mitochondria, suggesting an interaction with p38 MAPK in this compartment. Incubation of CESS cells with MKP-1-specific antisense oligonucleotides induces cell death not prevented by exogenous NGF. By contrast, overexpression of native MKP-1, but not of its catalytically impaired form, inhibits apoptosis induced by NGF neutralization in CESS cells.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant β-nerve growth factor, SB203580-p38 MAPK inhibitor, SB202474, K252a-Trk-A inhibitor, and proteasome inhibitor lactacystin were purchased by Calbiochem. Neutralizing rat anti-human NGF monoclonal antibodies (clone αD11) were kindly donated by Dr. A. Cattaneo (International School for Advanced Studies, Trieste, Italy) and always were used at 10 μg/ml. Rabbit anti-MKP-1 antibody and rabbit anti-ubiquitin were from Sigma. Rabbit anti-poly-(ADP-ribose) polymerase (PARP, H-250) and goat anti-actin antibody and immunoprecipitated rabbit anti-MKP-1 (V-15) antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho JNK, anti-JNK, anti-p44/42 MAPK, and anti-phospho-p44/42 MAPK were from New England Biolabs. Horseradish peroxidase-conjugated secondary antibodies were from Chemicon. Rabbit horseradish peroxidase-conjugated anti-EGFP antibody was from Clontech. ECL Plus Western blotting detection system was purchased from Amersham Biosciences. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) and actinomycin D were from Sigma. All of the other materials were purchased from either Sigma or Merck-Eurolab.

Cell Culture—CESS cell line was obtained from American Type Culture Collection and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin.

Reverse Transcription-PCR—Total RNA was isolated by using RNAFast reagent (Molecular Systems). Approximately 1 μg of RNA was reverse-transcribed in a total volume of 20 μl of specific buffer containing 50 ng of random hexamers, 1 mM dNTPs, 20 units of RNaseOUT (Invitrogen), and 20 units of avian myeloblastosis virus reverse transcriptase (Finnzymes) for 1 h at 37 °C. 2 μl of reverse transcription reaction was amplified by Dynazyme II DNA polymerase (Finnzymes) in the appropriate reaction buffer supplemented with 2 mM $MgCl_2$ and 1 mM dNTPs using the following primers: 5′-GCG GGA AAT CGT GCG TGA CAT T-3′ and 5′-GAT GGA GTT GAA GGT AGT TTC GTG-3′ for β-actin; and 5′-CCG GAG CTG TGC AGC AAA-3′ and 5′-CTC CAC AGG GAT GCT CTT-3′ for *MKP-1*, yielding a PCR products of 234 and 282 bp, respectively. PCR products were separated on 2% agarose gels with 100-bp Ladder marker (Invitrogen) and visualized with ethidium bromide staining.

Plasmid Construction and Cell Transfection—The human full-length MAPK phosphatase-1 coding sequence was amplified by PCR with primers DUSP1-FW (5′-GCT AGC AGA TCT ATG GTC ATG GAA GTG GGC ACC-3′) and DUSP1-RV (5′-GCT AGC AAG GAT CCG CAG CTG GGA GAG GTC GTA ATG GG-3′) using total cDNA of CESS cell line as template. The PCR fragment was subcloned in pCR2.1 vector (Invitrogen), yielding the p14-DUSP1 plasmid. pMEC10R plasmid coding for MKP-1/EGFP fusion protein under the CMV promoter was constructed by ligating in pEGFP-C1 (Clontech) in-frame to the N terminus of EGFP, the 1.1-kb NheI fragment from p14DUSP1 containing the *MKP-1* sequence. Transiently transfected cells were obtained using the LipofectAMINE reagent (Invitrogen) by following manufacturer's instructions. Catalytically impaired form (Cys^{258} to Ser) of MKP-1 was obtained by using the QuikChange site-directed mutagenesis kit (Stratagene) with primers 5′-AGG GTG TTT GTC CAC AGC CAG GCA GGC ATT TCC-3′ and 5′-GGA AAT GCC TGC CTG GCT GTG GAC AAA CAC CCT-3′ and the pMEC10R plasmid as cDNA template. All of the products were sequenced by BigDye Terminator kit (Applied Biosystem).

Immunoprecipitation Analysis—For immunoprecipitation studies, 10^7 cells cultured in the presence of 10 μM lactacystin with or without 10 nM hrNGF were lysed in 10 mM Hepes, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 50 μg/ml leupeptin, 30 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.25% Triton X-100, and 200 μg of cell lysate (as assessed by Bradford assay) were precleared with protein A-Sepharose and immunoprecipitated with 2 μg/ml rabbit anti-ubiquitin or rabbit anti-MKP-1 or control IgG followed by protein A-Sepharose. The immunoprecipitates were washed twice with PBS, boiled in Laemmli sample buffer, run on 10% SDS-PAGE, blotted onto nitrocellulose filter, and stained with anti-MKP-1 antibody or anti-ubiquitin antibody. Reaction was detected with ECL.

Purification of Nuclear, Mitochondrial, and Cytosolic Fraction—10^8 cells were suspended in 5 mM Tris, pH 7.4, with 5 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM EGTA, pH 8.0, containing 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.7 μg/ml pepstatin for 30 min on ice, Dounce-homogenized, and centrifuged at 750 × *g* at 4 °C to obtain the nuclear fraction. The supernatant was further centrifuged at 10,000 × *g* for 30 min at 4 °C to obtain the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). For Western blot analysis, the nuclear and mitochondrial fractions were directly lysed in sample buffer, whereas the cytosolic fraction was vacuum-concentrated and subsequently suspended in sample buffer. The purity of each fraction was assessed by staining aliquots with Abs to HSP60 for mitochondria, to actin for cytosol, and to PARP for nuclei.

Immunoblot Analysis—2 × 10^6 cells were lysed on ice in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 μg/ml leupeptin, 30 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitor mixture (Sigma), and 0.25% Triton X-100, pH 7.4. Lysates were centrifuged at 15,000 × *g* for 10 min and stored at −80 °C for further analyses, and protein concentration was determined by Bradford assay. Equivalent amounts of proteins were diluted in Laemmli sample buffer, heated at 90 °C for 3 min, loaded on 8% (for PARP analysis) or 12% (for MKP-1 and p38 MAPK analysis) SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and incubated with 1 μg/ml of primary antibodies diluted in TBS-T containing 5% nonfat dry milk for 1 h at room temperature. After washing with TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:1000 in TBS-T containing 5% nonfat dry milk for 1 h at room temperature, rinsed with TBS-T, and developed in ECL reagent. When necessary, membranes were stripped by heating at 56 °C in 62.5 mM Tris-HCl, pH 6.7, with 100 mM 2-mercaptoethanol and 2% SDS.

Antisense Assays—CESS cells were plated in 6-well plates in serum-free medium at the concentration of 10^6cells/well. Phosphorothioate oligonucleotides (MWG Biotech) were used for all of the antisense transfection experiments. Appropriate amounts of the oligonucleotides were diluted in 200 μl of serum-free medium. 5 μl of LipofectAMINE were added to each tube and incubated at room temperature for 30 min to allow LipofectAMINE reagent-DNA complex formation. The transfection mixtures were added to the wells and incubated for 5 h in serum-free medium. 10% fetal bovine serum then was added, and incubation was prolonged for different times (24–72 h). The cultures were washed in PBS and lysed as described above. The phosphorothioate oligonucleotides used were as follows: *p38 MAPK* antisense, 5′-gtC TTG TTC AGC TCC tgc-3′; *p38 MAPK* sense, 5′-gcA GGA GCT GAA CAA gac-3′; *p38 MAPK* scrambled, 5′-tgC TTA GTT CTC GTC cgc-3′ (28); *MKP-1* antisense, 5′-ccC ACT TCC ATG ACC Atg g-3′; *MKP-1* sense, 5′-ccA TGG TCA TGG AAG Tgg g-3′; and *MKP-1* scrambled, 5′-gcA GGA CGT GCT AGA ggg-3′.

Survival Assay—Approximately 5 × 10^3 cells/well were seeded in triplicate onto 96-well plates in RPMI 1640 medium and incubated for 24 h with 10 μM phosphorothioate anti-*MKP-1* or control oligonucleotides. NGF then was added at the final concentration of 10 nM, and cells were incubated for an additional 48 h. Detection of MTT reduction was performed as described previously (29). MTT was added to a final concentration of 0.5 mg/ml for 4 h. 100 μl of lysis buffer (20% SDS, 50% *N*,*N*-dimethylformamide, pH 7.4) and 100 μl of isopropyl alcohol were added to each well, and colorimetric reaction was measured by spectro-

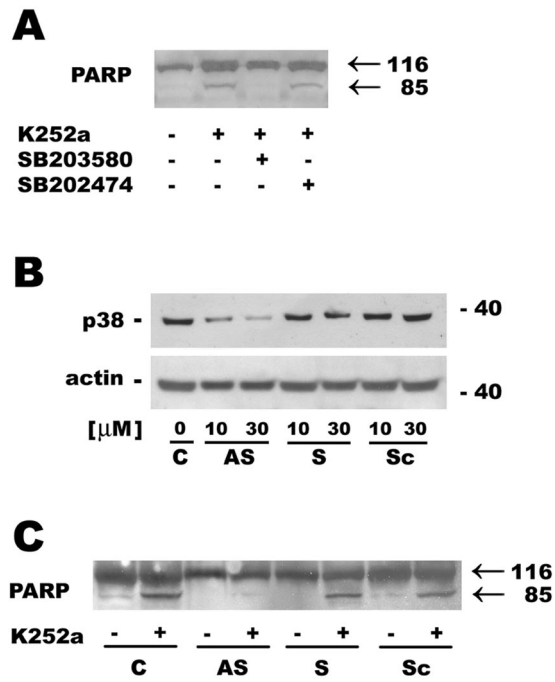

FIG. 1. **Inhibition of p38 MAPK by SB203580 or specific antisense oligonucleotides prevents caspase activation and apoptosis of CESS cells following NGF withdrawal.** *A*, CESS cells were incubated for 6 h in serum-free medium with 100 nM K252a in the presence or absence of 25 μM SB203580 or the inactive SB202474, lysed, and immunoblotted with anti-PARP antibodies. PARP cleavage, indicating activation of caspase-3-like enzymes, induced by K252a was inhibited by SB203580. Results from one experiment of three performed are shown. *B*, CESS cells were transfected in serum-free medium with 10 or 30 μM of specific antisense oligonucleotides to *p38 MAPK* (*AS*) or with the same concentrations of sense (*S*) or scrambled (*SC*) oligonucleotides as control for 48 h, lysed, and blotted with anti-p38 MAPK antibodies or with actin as protein-loading control. Antisense oligonucleotides specifically decreased p38 MAPK protein synthesis. Results from one experiment of three performed are shown. *C*, CESS cells were transfected with 30 μM AS, S, or SC oligonucleotides for 48 h, washed, and cultured for 6 h with 100 nM K252a. PARP cleavage induced by K252a was inhibited by antisense oligonucleotides but not by sense or scrambled oligonucleotides. Results from one experiment of three performed are shown.

photometric analysis by using a 570-nm filter.

Immunofluorescence Analysis—For immunofluorescence analysis, CESS cells were transiently transfected with pEMC10R or vector alone for 24 h, washed in PBS, and plated on poly-L-lysine-sensitized glass slides (Lab-Tek Chamber Slide, Nalge Nunc International) in serum-free medium. Cells were treated or not with 10 nM NGF for 30 min at 37 °C, labeled with 25 nM Mitotracker (Molecular Probes, Inc., Eugene, OR), washed twice with PBS, and fixed with 4% paraformaldehyde. After washing with PBS, nuclei were stained with Hoechst (Molecular Probes) and slides were analyzed by a confocal laser-scanning microscope.

RESULTS

Relevance of p38 MAPK Activation in Cell Apoptosis Induced by NGF Withdrawal—The $sIgG^+$ CESS B cell line originates from an antigen-selected somatically hypermutated, proliferating B lymphocyte, a stage ontogenetically close to that of memory B cells. Similar to memory B lymphocytes, CESS cells spontaneously produce NGF, express high and low affinity NGF receptors, and utilize NGF as an autocrine survival factor. Inhibition of endogenous NGF activity through neutralizing antibodies to NGF or use of the specific Trk-A inhibitor K252a (30) induces apoptotic cell death via a p38 MAPK-mediated Bcl-2 phosphorylation, an event that induces cytochrome *c* release and caspase activation (1, 4). Fig. 1, *panel A*, shows that SB203580, a specific inhibitor of p38 MAPK (31), is able to inhibit the apoptotic effect of NGF neutralization on

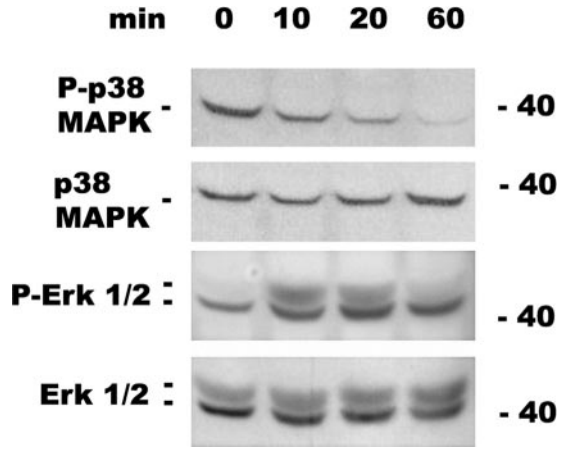

FIG. 2. **NGF induces dephosphorylation of p38 MAPK.** CESS cells were cultured in serum-free medium for 4 h and incubated with 10 nM hrNGF for the indicated times. Cell lysates were blotted and immunostained with rabbit anti-human phosphorylated p38 MAPK and with rabbit anti-human p38 MAPK, with rabbit anti-human-phosphorylated ERK, and with rabbit anti-human ERK as indicated. NGF promptly induced ERK activation and sustained dephosphorylation of p38 MAPK. Results from one experiment of three performed are shown.

CESS cells measured as caspase-3-like-mediated cleavage of PARP, whereas the inactive molecule SB202474 did not. The involvement of p38 MAPK in apoptosis induced by NGF neutralization was revealed also by using antisense oligonucleotides specific to *p38 MAPK* (28). CESS cells were transfected for 48 h with 10 or 30 μM anti-*p38 MAPK*-specific oligonucleotides or with the same concentrations of sense or scrambled oligonucleotides as control. Fig. 1, *panel B*, shows that antisense oligonucleotides induced a decrease of p38 MAPK synthesis with a maximum effect at a concentration of 30 μM (≈60% decrease). Next, CESS cells were again transfected with 30 μM antisense oligonucleotides and incubated with 100 nM K252a as NGF-neutralizing agent for 6 h. The cells were then lysed and blotted with anti-PARP antibodies as a measure of caspase activation. Fig. 1, *panel C*, shows that antisense oligonucleotides to *p38 MAPK* completely abolished PARP cleavage induced by K252a incubation, whereas sense or scrambled oligonucleotides did not. The above data confirm that p38 MAPK activation is necessary in cell apoptosis induced by NGF neutralization and support the contention that endogenous NGF exerts its survival factor function through a pathway involving p38 MAPK deactivation.

NGF Induces p38 MAPK Dephosphorylation and MKP-1 Expression—Stimulation of B lymphocytes with NGF induced tyrosine phosphorylation and activation of the two isoforms, p42 and p44, of Erk (32) as well as p38 MAPK dephosphorylation (4). To study the effect of NGF on phosphorylation status of MAPK, we cultured CESS cells in serum-free conditions for 4 h and stimulated them with 10 nM hrNGF for different times (from 10 min to 1 h). The phosphorylation status of ERK, p38 MAPK, and JNK was then studied by immunoblot analysis with antibodies specific for the phosphorylated enzymes. Fig. 2 shows that, under these experimental conditions, the activated forms of p38 MAPK and ERK were detected in unstimulated cultures. Activated JNK was never detected (data not shown). NGF stimulation had opposite effects on p38 and ERK, because it promptly induced p38 MAPK dephosphorylation, whereas ERK was instead strongly activated. 1 h after NGF stimulation, p38 MAPK continued to be dephosphorylated, whereas ERK phosphorylation kept returning to the base-line level. These results suggest the action of specific MAPK phosphatases with different activity toward ERK and p38 MAPK and are consistent with data generated using memory B lymphocytes (4).

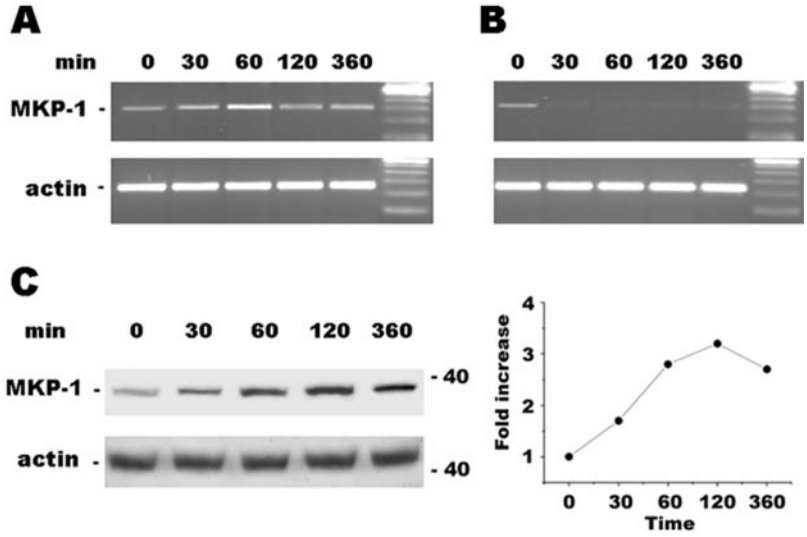

FIG. 3. **NGF induces *MKP-1* gene expression and protein synthesis by increasing transcriptional activity.** *A* and *B*, CESS cells were cultured for the indicated times with 10 nM hrNGF (*A*) or with 10 nM hrNGF in the presence of 3 μg/ml actinomycin (*B*), and mRNA was extracted. cDNA, obtained by retrotranscription, was amplified by using specific primers for *MKP-1* or for actin. NGF-induced *MKP-1* gene expression reached a peak after 1-h stimulation and returned to basal levels after 2 h. The induction of *MKP-1* mRNA was completely prevented by the addition of actinomycin, indicating an increase of transcriptional activity. *C*, CESS cells were incubated with 10 nM hrNGF for the indicated times. Cell lysates were blotted with anti-MKP-1 antibodies and with anti-actin antibodies as protein-loading control. NGF induced an increased synthesis of MKP-1 starting as soon as 30 min after stimulation and stable for up to 6 h. Results from one experiment of three performed are shown.

Among MAPK phosphatase family members known to deactivate p38 MAPK (33), MKP-1 and MKP-3 were reported to be induced by NGF in embryonic sympathetic neurons and in PC12 cells (34). Whereas MKP-3 interacts preferentially with ERK MAPK (35), it has been reported that JNK and p38 MAPKs were preferentially inactivated by MKP-1 with the following order of affinity, p38 MAPK > JNK > ERK (21). Furthermore, MKP-1 is encoded by an immediate early gene and is rapidly induced by many of the stimuli that activate MAPKs (20). For these reasons, we focused our attention on MKP-1.

To investigate whether NGF is able to activate *MKP-1* gene expression, CESS cells were starved from serum for 2 h and cultured in the presence or absence of 10 nM hrNGF for different times. mRNA was extracted, and *MKP-1* gene expression was analyzed by PCR with specific oligonucleotides. Fig. 3 *panel A* shows that *MKP-1* gene was rapidly induced in CESS cells after 30 min of incubation with exogenous NGF, reached a maximum expression after 1 h, and returned to the prestimulation levels after 6 h. Pretreatment of cells with 3 μg/ml actinomycin D completely blocked the induction of *MKP-1* mRNA in the presence of NGF (Fig. 3, *panel B*), thus indicating a transcriptional regulation, rather than mRNA stabilization mechanism. We next examined the time-dependent effect of NGF on MKP-1 protein levels. CESS cells were cultured in serum-free conditions in the presence or absence of 10 nM hrNGF for different times, lysed, and analyzed by Western blot analysis with specific antibodies. Fig. 3, *panel C*, shows that MKP-1 protein was constitutively expressed by CESS cells but NGF was able to increase expression up to 1.5-fold as early as 30 min after stimulation, reaching a peak (>3-fold increase) after 2 h. The increased levels of protein were stable up to 6 h after NGF stimulation. These data suggest that, in basal conditions, the production of endogenous NGF contributes to continuously sustain MKP-1 gene expression and protein synthesis.

NGF Decreases Degradation of MKP-1 through Proteasome Pathway—The early detection and the sustained levels of NGF-induced MKP-1 protein suggests that, in addition to the transcriptional regulation of *MKP-1* gene expression, NGF can stabilize MKP-1 protein by inhibiting its degradation. Recently, it has been demonstrated that MKP-1 is degraded via the proteasome pathway (36). To investigate the effect of NGF on MKP-1 degradation, we cultured CESS cells in the presence of the proteasome inhibitor lactacystin with or without 10 nM hrNGF for different times (1–6 h). Cells were lysed and immunoprecipitated with rabbit anti-ubiquitin antibodies or with rabbit anti-MKP-1 or rabbit IgG as control. Anti-ubiquitin-immunoprecipitated proteins were blotted and stained with anti-MKP-1 antibodies. Anti-MKP-1-immunoprecipitated proteins were blotted and stained with anti-ubiquitin antibodies. Western blot analysis was also performed with the same anti-MKP-1 antibodies to detect the level of intact MKP-1. Fig. 4 shows that ubiquitinated MKP-1 molecules were increased in untreated cultures in comparison with NGF-stimulated cultures. These results indicate that, with its combined action on mRNA as well as protein levels, endogenous NGF contributes to maintain adequate amounts of active MKP-1 in CESS cells.

Modulation of MKP-1 Protein Levels Changes the Phosphorylation Status of p38 MAPK—Because exogenous NGF increases MKP-1 synthesis and stability, we wanted to investigate whether NGF-neutralizing agents are able to modulate MKP-1 expression and whether the latter modulation is temporally related to the phosphorylation status of p38 MAPK. CESS cells were cultured in the presence of neutralizing antibodies to NGF for 12 h or with 100 nM K252 for 4 h, lysed, and blotted with anti-MKP-1 antibodies. Fig. 5, *panel A*, shows that incubation of cells with the above NGF-neutralizing agents inhibited the constitutive expression of MKP-1. In these experimental conditions, phosphorylation of p38 MAPK increased, whereas ERK phosphorylation decreased. Transfecting CESS cells with *MKP-1*-specific antisense oligonucleotides (37) yielded similar results. Fig. 5, *panel B*, shows that although p38 MAPK activation was increased under *MKP-1* antisense oligonucleotides treatment, ERK activation was slightly downmodulated, suggesting that basal ERK activation escapes MKP-1 regulation and that another phosphatase (PP-2a) is possibly responsible for its dephosphorylation (38). However, these results clearly show that MKP-1 is involved in p38 MAPK deactivation in CESS cells and suggest its relevant role in NGF-dependent cell survival.

MKP-1 Is Involved in NGF-mediated Survival Activity—To investigate the role of MKP-1 in the survival-promoting function of NGF, we performed two different experiments. In the first series of experiments, we studied whether forced expression of MKP-1 could prevent apoptosis induced by NGF withdrawal in CESS cell line. *MKP-1/EGFP* and a catalytically inactive form of *MKP-1/EGFP* (23) were cloned in pMEC10R plasmid under the CMV minimal promoter, and CESS cells were transiently transfected with these constructs or with

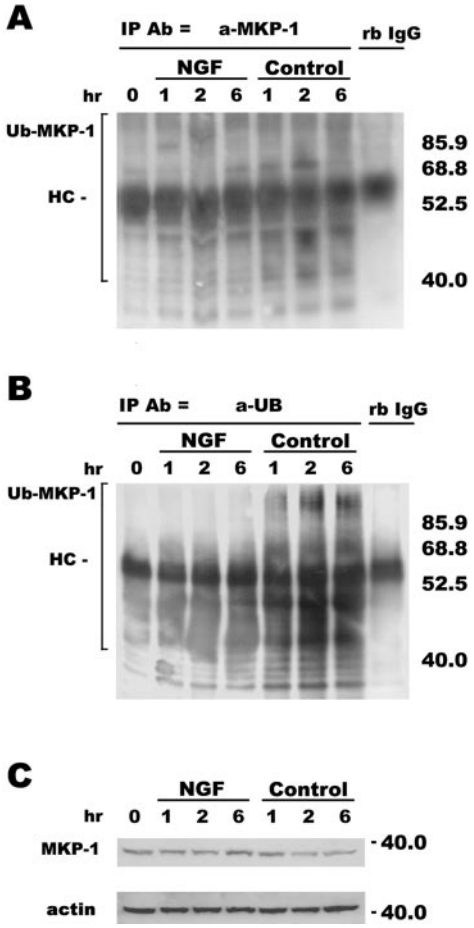

FIG. 4. **NGF inhibits MKP-1 degradation through the proteasome pathway.** 10^7 CESS cells were cultured in serum-free medium for the indicated times in the presence of 10 μM lactacystin with or without 10 nM hrNGF and lysed. Equal amounts of proteins from each sample were immunoprecipitated (*IP*) with anti-ubiquitin (*a-UB*) antibodies or with control Ig or with anti-MKP-1 antibodies and blotted. *A*, anti-ubiquitin-immunoprecipitated proteins were blotted and stained with anti-MKP-1 antibodies. *B*, anti-MKP-1-immunoprecipitated proteins were blotted and stained with anti-ubiquitin antibodies. *HC*, IgG heavy chain. *C*, Western blot analysis with the same anti-MKP-1 antibodies was also performed. Ubiquitinated MKP-1 molecules were increased in untreated cultures in comparison with NGF-stimulated cultures. Results from one experiment of three performed are shown. *Ab*, antibody; *rb*, rabbit.

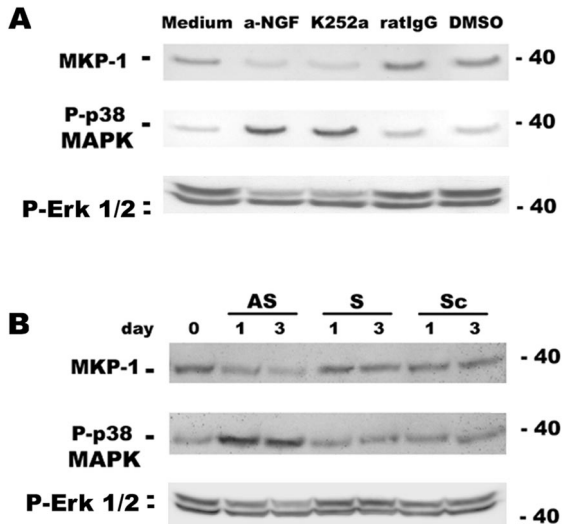

FIG. 5. **Inhibition of MKP-1 synthesis by NGF-neutralizing agents or specific antisense oligonucleotides induces phosphorylation (*P*) of p38 MAPK.** *Panel A*, CESS cells were cultured in serum-free medium for 4 h with 10 μg/ml of neutralizing rat anti-NGF monoclonal antibody or 10 μg/ml rat IgG as control with 100 nM K252a or equal volume of Me_2SO (*DMSO*) as control vehicle. The cells were lysed and immunoblotted with anti-MKP-1 antibodies with anti-phosphorylated p38 MAPK and with anti-phosphorylated ERK. NGF-neutralizing agents, which decrease MKP-1 protein levels, induce p38 MAPK activation as well ERK dephosphorylation. Results from one experiment of three performed are shown. *Panel B*, CESS cells were transfected with 10 μM anti-*MKP-1*-specific oligonucleotides (*AS*) or the same concentration of specific sense (*S*) or scrambled (*Sc*) oligonucleotides as control. After 1 or 3 days of treatment, MKP-1 synthesis decreased up to 60% while p38 MAPK was strongly activated. In contrast, ERK activation was slightly down-modulated. Results from one experiment of three performed are shown. *a-NGF*, anti-NGF.

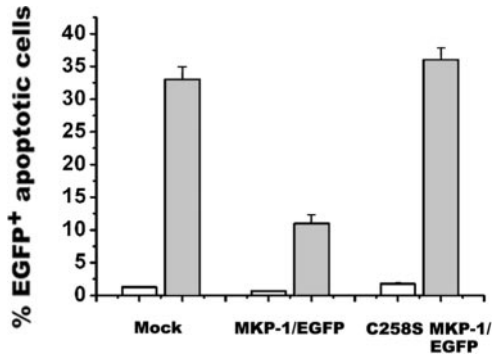

FIG. 6. **Overexpression of MKP-1 protects cells from the apoptosis induced by NGF neutralization.** CESS cells were transiently transfected with a plasmid coding for a MKP-1/EGFP fusion protein with a catalytically impaired "C258-S" MKP-1/EGFP and with EGFP alone as control (*Mock*) under CMV promoter for 24 h, washed, and lysed. EGFP/MKP-1 (active or inactive) fusion proteins were detected by immunoblotting with anti-EGFP antibodies (data not shown). CESS cells were transfected as above for 24 h and cultured in serum-free medium with 100 nM K252a for 6 h. Cells were then washed, stained with PE-annexin V, and analyzed by cytofluorimeter. Results are expressed as percentage of apoptotic cells (PE annexin V^+) over the whole transfected ($EGFP^+$) population. Cells expressing the MKP-1/EGFP fusion protein did not undergo apoptosis following K252a incubation, whereas cells expressing the catalytically inactive MKP-1/EGFP or EGFP alone did. Forced expression of active MKP-1 is able to prevent the effect of NGF neutralization upon apoptosis.

EGFP alone as control. After 48 h, cells were treated with 100 nM K252a for 6 h and apoptosis of transfected fluorescent cells was recorded as percentage of PE-annexin V-positive cells by cytofluorimetric analysis. Fig. 6 shows that cells expressing the MKP-1/EGFP fusion protein did not undergo apoptosis following K252a incubation, although control-inactive MKP-1/EGFP- or EGFP-expressing cells did, indicating that forced expression of MKP-1 is able to prevent the effect of NGF neutralization on apoptosis.

In the second series of experiments, we used antisense oligonucleotides to inhibit *MKP-1* gene expression and evaluated the survival of transfected cells. CESS cells were incubated in serum-free medium with antisense oligonucleotides to *MKP-1* or sense or scrambled oligonucleotides as control for 72 h in the presence or absence of 10 nM hrNGF. At the end of incubation, the amount of surviving cells was quantitated by MTT uptake and reduction by spectrophotometric analysis using a 570-nm filter as described previously (29). Table I shows that NGF is able to increase the survival of untransfected CESS cells in serum-free conditions. Antisense oligonucleotides to *MKP-1* strongly decreased cell survival, but the addition of NGF to antisense-transfected cells could not prevent cell death. These data strongly suggest that the transcriptional activation of *MKP-1* gene is crucial in maintaining the survival of CESS cells, being part of the metabolic pathway triggered by NGF.

NGF Induces Mitochondrial Localization of MKP-1 Protein—Mitochondrial localization of activated p38 MAPK and JNK/

TABLE I
Effect of MKP-1-antisense oligonucleotides on NGF-mediated survival of CESS cells

CESS cells were incubated in serum-free medium conditions in the presence or absence of the oligonucleotides for 72 h. hrNGF (10 nM) was added in selected cultures 24 h after transfection. Survival of cells was evaluated by MTT reduction assay. Data are expressed as mean ± S.D. of triplicate cultures. Results from one representative experiment of three performed are shown. Exogenous NGF is not able to prevent cell death induced by serum deprivation in the presence of *MKP-1*-specific antisense ODNs. ODNs, oligonucleotides.

	MTT reduction (Exp/SP*100) in the presence of			
	Medium	Antisense ODNs	Sense ODNs	Scrambled ODNs
Medium	100	46.1 ± 0.8	89.3 ± 1.7	94.3 ± 0.9
NGF	138.3 ± 4.8	45.2 ± 1.9	129.6 ± 3.4	132.7 ± 4.2

SAPK, induced by appropriate stimuli, was described in memory B lymphocytes and human U937 monocytic cell line, respectively (4, 39). In this compartment, the activated enzymes bind and phosphorylate Bcl-2 or Bcl-X_L protein, an event that triggers the apoptotic process. Because both p38 MAPK and JNK/SAPK represent the most relevant substrates for MKP-1 action, we asked whether MKP-1 could localize also in the mitochondrial compartment in CESS cell line. Mitochondrial, nuclear, and cytosolic fractions from 10^8 CESS cells, cultured in the presence or absence of 10 nM hrNGF for 30 min, were purified and blotted with anti-MKP-1 antibodies. To evaluate the purity of subcellular fractionation, the above fractions were also stained with anti-actin (cytosolic), anti-HSP60 (mitochondria), and anti-PARP (nuclear) antibodies. Fig. 7 *panel A* shows that, besides the nuclear compartment, MKP-1 is detected also in the mitochondrial compartment in CESS cell line, even in the absence of stimuli. However, NGF increased the amount of mitochondrial MKP-1.

To confirm these data, we transiently transfected CESS cells with a plasmid coding for a MKP-1/EGFP fusion protein or EGFP alone as control. 48 h after transfection, cells were cultured in the presence or absence of NGF for 30 min and labeled with Mitotracker (a rhodamine-like mitochondrial dye). After washing, the cells were attached on poly-L-lysine-sensitized glass slides and stained with a nuclear dye. Transfected (green fluorescent) cells were analyzed by confocal laser-scanning microscopy. Fig. 7, *panel B*, shows that, after addition of NGF, MKP-1/EGFP protein can be detected in the mitochondrial compartment (*yellow spot*) in amounts higher than those of unstimulated cultures.

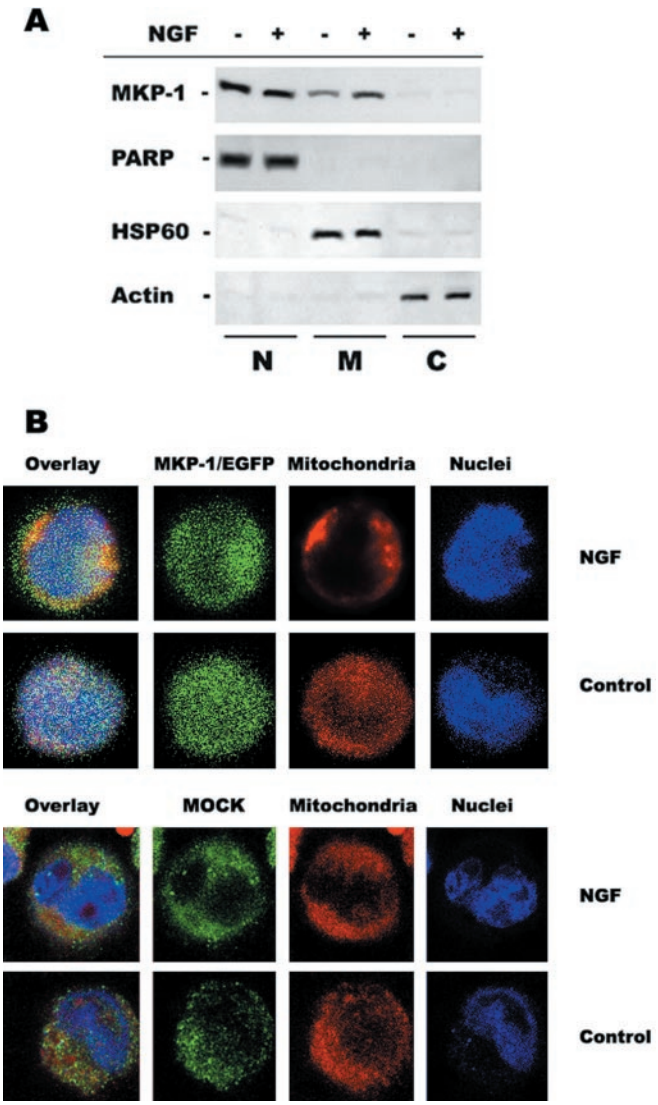

FIG. 7. **NGF induces mitochondrial translocation of MKP-1.** *Panel A*, mitochondrial (*M*), nuclear (*N*), and cytosolic (*C*) fractions of CESS cells, cultured in the presence or absence of 10 nM hrNGF for 30 min, were immunoblotted with anti-MKP-1 antibodies. Aliquots from the same preparation were blotted with anti-PARP antibodies, with anti-HSP60, and with anti-actin antibodies to assess the purity of subcellular fractions. The mitochondrial amounts of MKP-1 increased in NGF-treated cultures. *Panel B*, CESS cells were transiently transfected with a plasmid coding for a MKP-1/EGFP fusion protein or EGFP alone as control (*MOCK*) under CMV promoter for 24 h, washed, and plated on poly-L-lysine-sensitized glass slides in serum-free medium. Cells were then cultured in the presence or absence of 10 nM hrNGF and labeled with 25 nM Mitotracker at 37 °C for 30 min. Nuclei were stained with Hoechst dye., and the slides were analyzed by a confocal laser-scanning microscope. Mitochondrial localization of MKP-1/EGFP fusion protein defined by *yellow spots* indicating overlap of fluorescein and rhodamine was strongly increased in NGF-treated cultures.

DISCUSSION

NGF is a classical survival factor, essential for a large number of cell types, including neurons, keratinocytes, and memory B lymphocytes. In all of these cells, discontinuation of the NGF signal causes apoptotic death that critically involves mitochondria with alterations of proteins of the Bcl-2 family (3, 40–42). In memory B lymphocytes and in the lymphoblastoid CESS cell line, we described that, upon neutralization of endogenous NGF, p38 MAPK in its active form rapidly translocates onto mitochondria, specifically interacts with Bcl-2, and phosphorylates it in the Ser^{87} and Thr^{56} residues, an event that decreases the anti-apoptotic potential of Bcl-2, thus allowing cytochrome *c* release and apoptotic cell death (4). Bcl-2 phosphorylation by activated p38 MAPK is a crucial event in apoptosis induced by NGF neutralization because cells expressing the loop-deletional mutant cDNA construct, Bcl-2 Δ40–91, were completely resistant to apoptosis induced by NGF withdrawal (1). The role of activated p38 MAPK in Bcl-2 phosphorylation and apoptosis caused by NGF neutralization was further demonstrated by its ability to induce cytochrome *c* release in isolated mitochondria and by inhibition of cell death operated by p38 MAPK inhibitors or p38 MAPK-specific antisense oligonucleotides (data reported in Fig. 1) (4).

Conversely, when NGF is added to factor-starved cells, p38 MAPK is promptly deactivated and, hence, Bcl-2 phosphorylation is inhibited (4). In this paper, we characterize the NGF-induced phosphatase MKP-1, which is responsible for active p38 MAPK dephosphorylation in CESS cell line and which plays a relevant role in NGF-mediated autocrine circuit of survival. This NGF-induced phosphatase is a dual specificity phosphatase that selectively dephosphorylates tyrosine and threonine residues on MAPKs, being particularly active toward

p38 MAPK and JNK/SAPK substrates (21). In fact, the addition of NGF to serum-starved cell cultures induces a rapid dephosphorylation of p38 MAPK, which is maintained up to 1 h after NGF stimulation. Conversely, ERK phosphorylation, induced by upstream kinases activated by Trk-A signaling, starts to decrease 1 h after NGF stimulation. We interpreted the above data as the result of an NGF-induced MAPK phosphatase with greater affinity toward p38 MAPK than ERK and hypothesized that MKP-1 was the most probable candidate.

The up-regulation of MKP-1 gene expression induced by NGF was reported also in other cellular systems, such as dissociated embryonic sympathetic neurons or fibroblasts transfected with the high affinity NGF receptor Trk-A (34). It is believed that activation of Trk-A is crucial for *MKP-1* gene expression, independently of the cellular origin or type on which the Trk-A receptor is active (34). NGF increases MKP-1 protein levels through both an active transcriptional mechanism and a decrease of protein degradation. The induction of *MKP-1* mRNA by NGF was rapid (within 30 min) and completely blocked by actinomycin, consistent with data reported in other experimental systems (37, 43). In fact, *MKP-1* is considered an early response gene (20) whose transcriptional activity is controlled via a strong block of elongation in the exon I of the gene (44). Epidermal growth factor is able to remove this block in neuroendocrine cells (44), and activation of the p42/p44 MAPK cascade as well as that of protein kinase Cϵ is involved in the induction of *MKP-1* gene (27, 44). In B lymphocytes and in lymphoblastoid cell lines, the autophosphorylation of Trk-A induced by NGF strongly activates the p42/p44 MAPK cascade (our data and Ref. 45), delineating a pathway for *MKP-1* gene induction. Consistently, a specific Trk-A inhibitor is able to block *MKP-1* gene expression (Fig. 5).

Although *MKP-1* mRNA reached a peak after 1 h and returned to basal level after 2 h of stimulation with NGF, the increased levels of MKP-1 protein, evident already 30 min after stimulation, were stable for several hours. It is known that MKP-1 is degraded through the classical proteasome pathway (36, 46) and the reported half-life of MKP-1 is not longer than 45 min (36). The stable (up to 6 h) levels of MKP-1 protein induced by NGF stimulation are related to a reduced ubiquitin binding and degradation of MKP-1 protein, probably because of its phosphorylation, again possibly operated by ERK, as suggested by Brondello *et al.* (36) and Chen *et al.* (47). This latter point is actually under investigation in our laboratory.

Previous reports show the importance of MKP-1 in the regulation of apoptosis in various cells (48). For example, spontaneous or conditional expression of MKP-1 in cancer cell models is paralleled by their resistance to apoptosis (48–50). MKP-1 was also involved in the anti-apoptotic effect of retinoids in mesangial cells incubated with H_2O_2 (43) as well as in the restriction of TNF-induced apoptosis in mesangial cells (51). In all of these experimental systems, the anti-apoptotic effect of MKP-1 was related to its ability to dephosphorylate p38 MAPK and/or JNK/SAPK.

In CESS cells, inhibition of MKP-1 synthesis by specific antisense oligonucleotides, temporally related to activation of p38 MAPK, is followed by reduced cell viability (Table I). NGF neutralization decreases MKP-1 expression and induces p38 MAPK activation, thus mimicking the effect of antisense oligonucleotides. For these reasons, we thought that NGF-induced MKP-1 plays an essential role in NGF-dependent survival of CESS cells, mainly through a continuous p38 MAPK dephosphorylation. This hypothesis was confirmed by lack of survival-promoting action of NGF in serum-starved cells transfected with specific anti-*MKP-1* oligonucleotides as well as by lack of pro-apoptotic effect of NGF-neutralizing agent in cells overexpressing MKP-1. All of these functional data allow us to define MKP-1 as a major actor in the survival function of NGF in lymphoblastoid cells.

Here, we first report a mitochondrial localization of MKP-1. Although scanty amounts of the enzyme can be shown even in unstimulated cultures, NGF induces a conspicuous translocation of the enzyme in this compartment. In cells overexpressing MKP-1/EGFP fusion protein, the phenomenon can be easily monitored. This novel localization of MKP-1 should not be a surprise because it is known that at least three substrates of MKP-1, namely ERK, JNK, and p38 MAPK, were found in the mitochondrial compartment (4, 39, 52). The mitochondrial carriers of these kinases are not completely defined. An adapter protein, Sab (SH3BP5), was described as able to address JNK to mitochondria (53), but it can be hypothesized the involvement of Grb proteins (52), possibly shuttling between plasma membrane receptors and the apoptosis-operating machinery whose activity is modulated through phosphorylation/dephosphorylation of proteins involved in maintaining outer membrane mitochondrial permeability. However, we cannot rule out the involvement of other adapter proteins, such as HSP, able to transport kinases/phosphatases to mitochondria (53). Recently, the role of JNK and p38 MAPK on mitochondria has been defined in more detail and, besides Bcl-2/Bcl-X_L phosphorylation, activation of other pro-apoptotic members of Bcl-2 family (Bim, Bmp) has been reported previously (54) as well as phosphorylation of other ill-defined mitochondrial proteins (55).

The final result of JNK and p38 MAPK mitochondrial localization is the release of cytochrome *c* and SMAC/DIABLO (4, 39, 56). By contrast, ERK localization on mitochondria was instead reported as related to Bad phosphorylation through interaction with PKCϵ, resulting in a mitochondrial activity likely to be an inhibition of cell apoptosis (52). Although we do not know how ERK is transported to mitochondria (see above), it is conceivable that this compartmentalization increases in those conditions such as NGF stimulation, leading to strong ERK activation. MKP-1 translocation, in turn, can be driven by high concentrations of its substrate ERK. Post-translational modifications probably influence the interaction of MKP-1 with different mitochondrial proteins as suggested by preliminary results obtained in our laboratory, indicating that NGF induces a down-modulation of MKP-1 tyrosine phosphorylation. However, MKP-1 mitochondrial localization in NGF-stimulated cultures supports the hypothesis that the enzyme is ideally suited for dephosphorylation of p38 MAPK molecules activated following stimuli not strictly related to apoptosis induction. In other words, mitochondrial MKP-1 can play a "sentinel" role toward potentially dangerous activated p38 MAPK or JNK molecules. This hypothesis is strengthened by the occurrence of NGF-induced MKP-1 mitochondrial localization as well as in other cellular systems in which NGF performs an autocrine survival function, such as keratinocytes.[3]

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