Journal of Cellular Biochemistry 101:112–121 (2007)

Large Scale Genetic Screen Identifies MAP17 as Protein Bypassing TNF-Induced Growth Arrest

M.V. Guijarro, M.E. Castro, L. Romero, V. Moneo, and A. Carnero*

Experimental Therapeutics Program, Centro Nacional de Investigaciones Oncológicas (CNIO), Madrid, Spain

Abstract Although activated macrophages destroy cancer cells more effectively than normal cells, the ability to escape activated macrophages is a characteristic of tumor cells. One of the mechanisms responsible for the specific killing of tumor cells by macrophages is the production of the cytokine tumor necrosis factor (TNF) alpha. Therefore, resistance to TNF may provide such cancer cells a selective advantage against host elimination. With the aim of identifying genes with these properties we undertook a large scale genetic screen to identify genes able to bypass TNF-induced G1 arrest. We identified MAP17, a small 17 kDa nonglycosylated membrane protein that localizes to the plasma membrane and the Golgi apparatus. Ectopic expression of MAP17 in tumor cells prevents TNF-induced G1 arrest by impairing p21waf1 induction. However, expression of MAP17 does not inhibit TNF-induced apoptosis in Me180-sensitive tumor cells. The inhibition of TNF is specific since MAP17 does not alter the response to other cytokines such as IFNγ. As described in the *Xenopus oocyte* system, MAP17 increases the uptake of mannose in some cells, but this effect is not responsible for TNF bypass. We have also analyzed the expression of MAP17 mRNA in a panel of cell lines. MAP17 is expressed in 30% of cell lines of different origin. However, MAP17 mRNA expression did not correlate with TNF resistance. Our data indicates that although MAP17 expression might bypass TNF-induced growth arrest, it is not the only determinant of this response. J. Cell. Biochem. 101: 112–121, 2007.

Key words: genetic screening; TNF; MAP17

Tumor necrosis factor (TNF) is one of the most important cytokines produced by almost all cell types of the immune system [Beutler and Cerami, 1988; Vilcek and Lee, 1991]. TNF mediates a wide variety of biological effects that include activation of neutrophils and monocytes [Shalaby et al., 1985; Feinman et al., 1987]; mediation of the inflammatory response [Beutler and Cerami, 1988], endotoxic shock [Tracey et al., 1988], and cytostatic and/or cytotoxic effects in tumor cells in vitro and in vivo [Carswell et al., 1975; Sugarman et al., 1985; Haranaka et al., 1986; Wanebo, 1989; Balkwill et al., 1990; Hieber and Heim, 1994]. This range of biological activities depends on the cell type, stage of differentiation, and transformation state. It has been estimated that 40% of tumor cell lines are growth inhibited by TNF in vitro [Goeddel et al., 1986], but not normal cells from lung, colon, or fetal skin [Sugarman et al., 1985].

A variety of cellular targets and metabolic or molecular changes have been linked to the cytotoxic effects of TNF, which includes DNA fragmentation, changes in lipid metabolism, lipid peroxidation, and increases in prostaglandin E production. TNF can also induce the phosphorylation of the EGF receptor and molecules such as hsp 27 and hsp 70, NFκB and the eukaryotic initiation factor eIF 4E, activation of phospholipases A2 and D, sphingomyelinase and ICE protease. Overexpression of Hsp 27 and Hsp 70 proteins as well as Bcl2, E1B 19k, cathepsin D, MnSOD or B/c-sis genes as well as the activation of the PI3K-AKT pathway protect cells against TNF-induced cytotoxicity (for review see [Baker and Reddy, 1996; Schulze-Osthoff et al., 1998; Ozes et al., 1999; Aggarwal,

Grant sponsor: Spanish Ministry of Health; Grant number: FIS-02/0126; Grant sponsor: Fundacion Mutua Madrileña and Spanish Ministry of Education and Science; Grant number: SAF2005-00944.

*Correspondence to: A. Carnero, Experimental Therapeutics Program, Centro Nacional de Investigaciones Oncológicas (CNIO), Melchor Fernández Almagro, 3, 28029, Madrid, Spain. E-mail: acarnero@cnio.es

Received 14 July 2006; Accepted 6 September 2006

DOI 10.1002/jcb.21163

2000; Burow et al., 2000; Chen and Goeddel, 2002; MacEwan, 2002]). Although TNF shows well-documented apoptotic activity on tumor cells, it also produces cytostatic effects on other cell lines with concomitant G1 arrest [Jeoung et al., 1995; Harvat and Jetten, 1996; Shiohara et al., 1997; Merli et al., 1999]. In our previous work we demonstrated that the cyclin D1 oncogene overrides the cytostatic effect of TNF [Moneo et al., 2003]. However, other oncogenes such as ras, c-myc and E1A have been shown to induce cellular susceptibility to the cytotoxic action of TNF [Seliger et al., 1988; Fernandez et al., 1994; Chang et al., 1999].

In the present work we realize a genome wide genetic screening to identify genes that bypass TNF-induced cytostatic effects with the aim of identifying new genes with oncogenic potential. We found MAP17, a membrane-bound protein whose expression selectively blocks TNF-induced growth arrest by inhibiting p21 activation and pRB dephosphorylation.

MATERIALS AND METHODS

Cell Culture

A375 malignant melanoma cells, T47D ductal breast carcinoma, Me180 cervix carcinoma, HBL100 breast epithelial cells transformed with SV40, were obtained from American Type culture Collection (Manassa, VA). A375 and HBL100 were maintained in Dulbecco's modified Eagle's (Gibco), T47D in RPMI 1640 (Sigma Chemical Co, St. Louis, MO) and Me180 in McCoy's (Sigma) containing 10% fetal bovine serum (Sigma), penicillin, streptomycin and fungizone.

MAP17 full-length cDNA was cloned into pBabe puro and clones generated by stable gene transfer in A375, T47D, Me180, and HBL100. Numerous clones were selected and tested. All behaved similarly. For this work four representative clones were used. MAP17 full-length cDNA was also cloned in pWZLblast fused to GFP. To perform the gene transfer using ecotropic competent retroviruses, A375 cells were transfected with the murine receptor and a mass culture expressing the receptor used for the following experiments. When indicated, cells were treated with purified human recombinant TNF (Gibco-BRL). Cultures were selected when indicated with 75 μg/ml hygromycin (Calbiochem), 400 μg/ml G418 (Gibco), 2 μg/ml Puromycin (Calbiochem) or 0.5 μg/ml of Blasticidine (Calbiochem).

Retrovirus-Mediated Gene Transfer

Packaging LinXE cells were plated in a 10 cm dish, incubated for 24 h and then transfected by calcium phosphate precipitation with 20 μg of the retroviral plasmid (16 h at 37°C). After 48 h, the virus-containing medium was filtered (0.45 μm filter, Millipore) and supplemented with 8 μg/ml polybrene (Sigma) and an equal volume of fresh media. Target cells were seeded at 10^6 cells/10 cm dish and incubated overnight. For infections, the culture medium was replaced by the appropriate viral supernatant, the culture plates were centrifuged (1 h, 1,500 rpm) and incubated at 37°C for 16 h. The infected cell population was purified using the appropriate selection.

Generation and Transduction of the cDNA Library

mRNA was extracted from TNF-treated human cells. Aliquots of 2 μg of total mRNA were used for the generation of the library. OligodT primed cDNA was synthesized from the polyA+ mRNA and cloned into the EcoRI and XhoI sites of pMARXIIpuro [Hannon et al., 1999]. To generate the viral carriers of the library, samples of 5×10^6 LinXE ecotropic packing cells were plated per 10 cm dish, incubated during 24 h and transfected by calcium phosphate precipitation using 20 μg of retroviral plasmid. After 48 h, the virus-containing medium was filtered (0.45 μm filter; Millipore) and supplemented with 8 μg/ml polybrene and an equal volume of fresh medium. 10^6 A375 cells were infected with the appropriate viral supernatant and the culture plates were centrifuged (1 h, 1,500 rpm) and incubated at 37°C for 16 h. After 2 μg/ml puromycin selections, the stably transfected A375 were seeded at low density and treated with 200 nM TNF for 15 days. Colonies formed during this time were selected and grown independently.

In Vitro Recovery of Proviruses

It was performed as previously described in Carnero et al. [2000]. Briefly, total genomic DNA was extracted from each one of the TNF-resistant clones. To excise the proviruses, 10 μg of genomic DNA were digested with CRE recombinase (DNA final concentration 0.1 μg/μl)

for 3 h at 37°C, extracted with phenol/chloroform and ethanol precipitated. DNA was washed extensively with 70% ethanol and dissolved in 5 μl of water. Aliquots of 2–5 μg of total DNA were electroporated into DH10B-lactrfA bacteria and proviruses recovered as a fully functional retroviral plasmid from zeocin-resistant bacterial colonies.

RT-PCR

Total RNA was purified using the TRI-REAGENT (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed with 5 μg of mRNA using MMLV reverse transcriptase (Promega) and oligo dT primer according to the manufacturer's recommendations. The following primers were used to amplify regions: MAP17 forward 5′-CAGCCATGTCGGCCCTCA-3′ and reverse 5′-TTA TTTCACAGAAATTAGGGCC-3′; β-actine: forward 5′-AGGCCAACCGCGAGAAGATGAC-3′ and reverse 5′-GAAGTCCAGGGCGACGTAGCA-3′. cDNA was subjected to PCR and products were analyzed by 1% agarose gel electrophoresis.

Western Blot Analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by sonication in lysis-buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 2 mM Na_3VO_4, 150 mM NaCl, 20 mM $Na_4P_2O_7$, 100 mM NaF and complete protease inhibitor cocktail (Roche Molecular Biochemicals)). The protein content of the lysates was determined by the modified method of Bradford using bovine serum albumin (BSA) as a standard and the appropriate quantity of protein was then dissolved in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue (Sigma)). Samples were separated on 7.5–15% SDS–PAGE gels, transferred onto PVDF membranes (Immobilon-P, Millipore) and immunostained. The following primary antibodies were used: MAb anti-p21 (Santa Cruz 397, diluted 1:500), MAb anti-pRb phosphorylated (BD Pharmingem 554136, diluted 1:400), MAb anti-α-tubulin (Sigma 9026 1:10,000), and horseradish peroxidase-labeled rabbit anti-mouse (Promega diluted 1:5,000) and goat anti-rabbit (Calbiochem 401315, diluted 1:4,000) secondary antibodies. Proteins were visualized using the ECL detection system (Amersham Biosciences, Buckinghamshire, UK).

Flow-Cytometric Assay for Apoptosis

Me180 attached parental and MAP17 expressing cells were trypsinized, combined with cells floating in the media and centrifuged (1,000 rpm, 5 min) cells pelletes were washed with PBS 1× and then resuspended in 1× binding buffer (BD Pharmingen; 10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were stained using 5 μl Annexin V (BD Pharmingen) and 10 μl propidium iodide (Sigma) and incubated for 15 min in the dark. Samples were analyzed in a Beckton and Dickinson FACScalibur.

Mannose Transport Assays

For uptake curves, 10^5 logarithmically growing cells were seeded in 24 well plates and preincubated with 1 or 35 μM Mannose (Sigma) for 1 h at 37°C. Then, 50 μCi/ml of D-[2-^{3}H] Mannose (Amersham Biosciences) was added and incubated 4 h at 37°C. After washing to eliminate radioactivity, cells were lysed (50 mM Tris-HCl pH 7.5, 1% NP-40, 150 mM NaCl) and prepared for counting with an aqueous scintillation cocktail in Wallac 1414. For mannose dose-response, 10^5 logarithmically growing parental cells were seeded in 24 well plates and different concentrations of D-[2-^{3}H] Mannose were added. Cells were incubated 4 h at 37°C and then prepared for counting. In the case of TNFα dose-response, TNFα was dissolved in media containing 100 μM Mannose.

Doses-Response Treatments

Dose response to TNF (Gibco-BRL) and IFN-γ (Biosource) was generated by seeding 10^4 cells in 96 well plates in triplicate samples. After 24 h, medium was changed (day 0) and the indicated dose of the drug was added. After 4 days cells were fixed and stained with crystal violet (Sigma). After extensive washing, crystal violet was resolubilized in 15% acetic acid and quantified at 595 nm as a relative measure of cell number. Values are expressed as the percentage of cell growth. Untreated control cells represent 100% of cell growth. Zero percent refers to the number of cells at day 0.

RESULTS

MAP17 was Identified Screening for Inhibitors of TNF-Dependent G1 Arrest

To identify new regulatory proteins with the ability to influence tumoral outcomes we undertook a screen to identify genes that, when expressed at high levels, were capable of bypassing TNF-induced growth arrest. To that end we used the A375 human melanoma cell line. In this cell line, we previously showed that TNF induces p21waf1 protein, which binds to CDK complexes correlating with the inhibition of the activity of the complexes and concomitant G1 arrest [Moneo et al., 2003]. The overexpression of cyclin D1 in A375 cells makes them insensitive to TNF treatment with the recovery of CDK activity. We used this cellular system to identify new genes impinging in TNF-induced arrest.

A random cDNA library generated from total polyA+ RNA was cloned into the MMLV-based retroviral vector pMARXII [Hannon et al., 1999; Carnero et al., 2000]. This library contained $\sim 8 \times 10^6$ independent clones. The cDNA library was transfected into retrovirus packaging cells and replication-deficient viruses were infected into exponentially growing A375 cells. Following selection, $\sim 10^5$ cells/100 mm culture dish were plated and treated with 200 nM TNFα. After 2 weeks, colonies that had overcome TNF-induced G1 arrest were identified and the genomic DNA was extracted. Proviruses containing the cDNA were excised from the total genomic DNA and independent proviruses carrying individual cDNAs were retested through infection on A375 cells. Positive TNF effect-bypassing genes were selected, sequenced and identified by homology with databases (Fig. 1A).

We obtained 36 TNF resistant clones carrying cDNAs which ectopic expression bypasses TNF-induced growth arrest. Among the positive clones we recovered full length cDNAS from Pre B enhancing factor (PBEF) four times, Casein kinase ε (CK ε) three times and membrane associated protein 17 (MAP17, DD96) eight times. Another 15 clones contained cDNAs not comprising the full open reading frame and the rest, 6 clones, were yet unidentified sequences.

Among the genes with full ORF we selected MAP17 because no previous relation with TNF

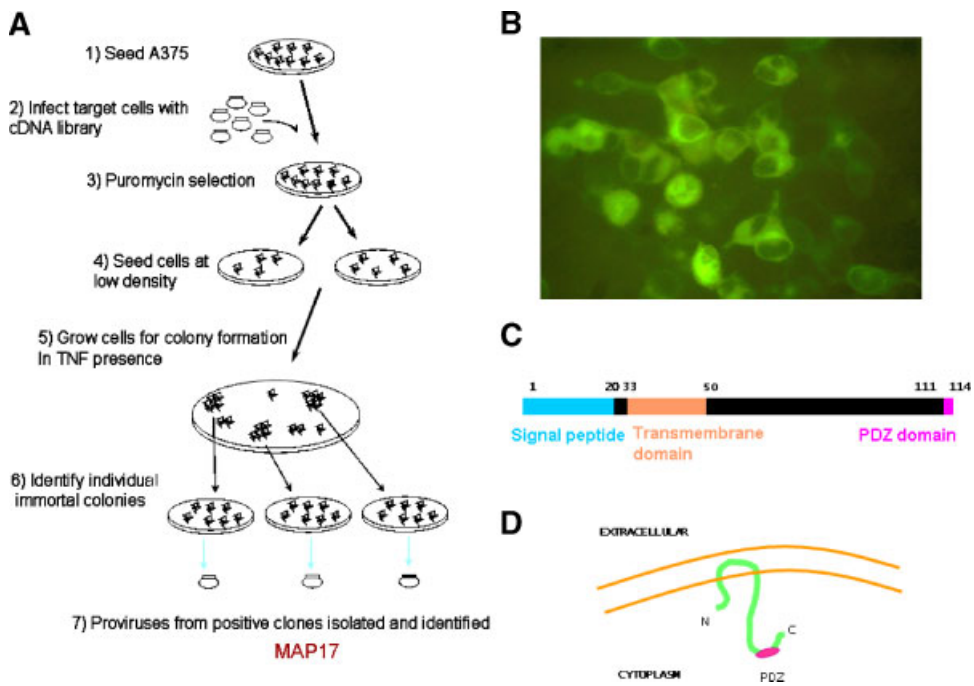

Fig. 1. MAP17 was identified screening for inhibitors of TNFα-dependent G1 arrest. **A**: Diagram of the screening and isolation of the protein. A375 cells were infected with a cDNA library, selected with puromycin and treated with TNFα. Then, individual clones were isolated and MAP17 identified. **B**: Localization of MAP17. Confocal imaging of HEK293 transiently transfected with MAP17. **C**: Schematic representation of the protein. MAP17 has 114 aminoacids and the structure prediction is showed. It has a signal peptide, a transmembrane domains and a PDZ domain. **D**: Possible localization of MAP17 inside the cell. Predicted disposition of MAP17 in the membrane, with both sides of the protein localized to the cytoplasm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

was previously reported. The sequence of MAP17 recovered from the screening included the full ORF matching 100% with the U21049 sequence reported by Kocher et al. [1995]. MAP17 is a 17 kDa nonglycosylated membrane protein that locates to the plasma membrane and the Golgi apparatus ([Blasco et al., 2003] and Fig. 1B). The protein sequence showed a 61-aminoacid hydrophilic amino-terminus coding a signal peptide, two transmembrane regions and a hydrophobic carboxi-terminus of 13 aminoacids coding a PDZ domain (Fig. 1C). MAP17 acts as an atypical anchoring site for PDZK1 and interacts with the NaPi-IIa/PDZK1 protein complex in renal proximal tubular cells [Pribanic et al., 2003]. The physiological role of MAP17 in proximal tubules is not known but stimulates specific Na-dependent transport of mannose and glucose in *Xenopus* oocytes [Blasco et al., 2003].

MAP17 Bypass TNF-Dependent G1 Arrest

Next, we explored the ability of the MAP17 expressing clones to bypass TNF-induced arrest. To that end, we generated clones of A375 melanoma cells ectopically expressing the full cDNA driven by the LTR promoter. We selected several clones and characterized the MAP17 mRNA expression by RT-PCR (not shown). Expression was confirmed by FAC scan and by immunohistochemistry and immunofluorescence (not shown).

We subjected the parental cells as well as the clones to increasing doses of TNF. Parental cells showed a constant decline of growth with increasing doses of TNF (Fig. 2A). However, all clones were resistant to TNF-induced growth arrest and kept growing even at high doses of TNF (Fig. 2A).

The effect of ectopic expression of MAP17 bypassing TNF was also observed on other cell line such as T47D where TNF also exerts cytostatic effects (Fig. 2A). However this effect was not universal, since in ME180, an ovarian carcinoma cell line where TNF induces cytotoxicity, MAP17 does not produce significant effects and the protection is minimal (Fig. 2A). Furthermore, the effect of other cytokines such as IFNγ was not altered by ectopic expression of MAP17 (Fig. 2B).

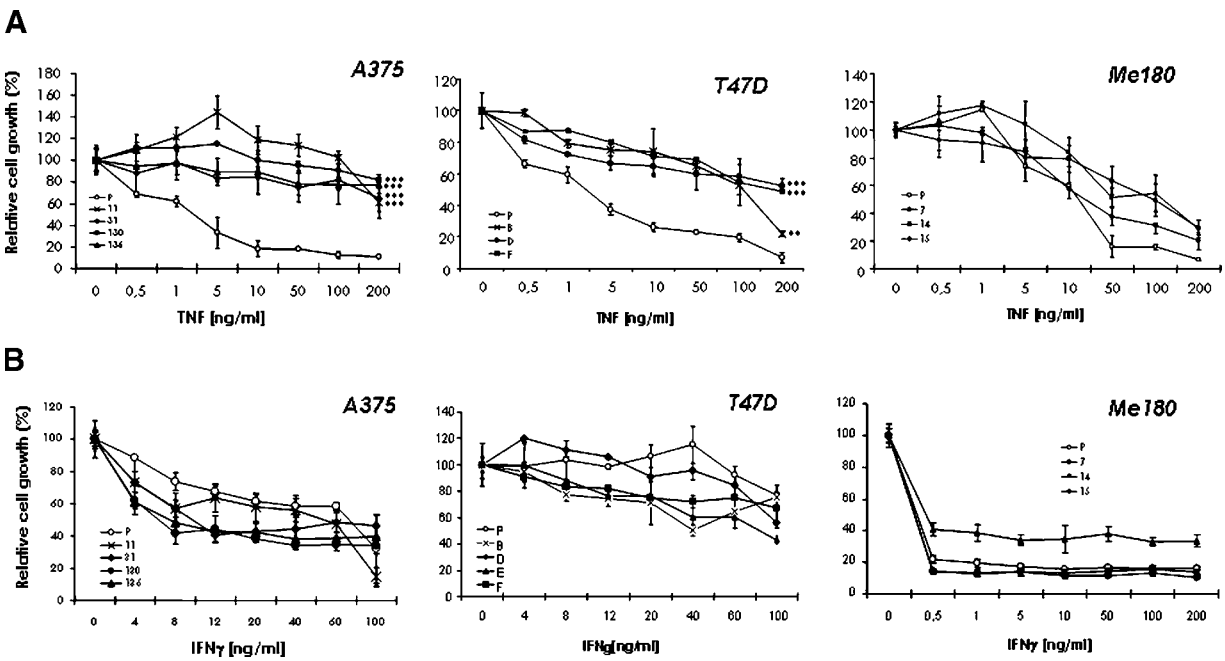

Fig. 2. **A**: MAP17 inhibits TNFα-induced growth arrest. Dose-response effect of TNFα treatment in parental cells and MAP17 expressing clones in A375, T47D, and Me180 cells. Values are expressed as the percentage of cell growth. Untreated control cells represent 100% of cell growth. The data are presented as mean from triplicate samples; bars, ± SD. One representative experiment out of three performed is shown. **B**: MAP17 does not alter IFNγ-induced growth arrest in A375 nor Me180. Dose-response effects of IFNγ treatment in the same cells as above. Values are also expressed as the percentage of cell growth. Parental A375, T47D and Me180 cells (P); MAP17 expressing clones (A375: 11, 31, 130, 136; T47D: B, D, F; Me180: 7, 13, 14). ANOVA test was used to compare the statistical significance of the differences among the survival curves of parental cells versus the clones. The study was made with the programme R 2.3.1 (R Development Core Team [2006]). Significant codes: '***' 0.001; '**' 0.01; '*' 0.05.

In previous work we demonstrated that TNF induces p21waf1 protein in malignant melanoma A375 cells by inhibiting the activity of CDK2 and CDK4 complexes. This inhibition leads to dephosphorylation of pRb and consequent G1 arrest. Overexpression of cyclin D1 in these cells makes them insensitive to TNF treatment with the recovery of CDK activity and sustained phosphorylation of pRb even in the presence of TNF [Moneo et al., 2003]. To explore whether the mechanism elicited by MAP17 to bypass TNFα-induced growth arrest was related to p21waf1 and pRb phosphorylation, we measured the effect of TNF on these proteins in the parental cells and in MAP17 expressing clones (Fig. 3A). As previously described, TNF induced the increase of p21waf in the parental cells with concomitant pRb dephosphorylation. However, in the different MAP17-expressing clones TNF was unable to induce p21waf1, and consequently pRb was maintained in a phosphorylated state, allowing cell proliferation (Fig. 3A).

In Me180, we tested whether the MAP17 expression altered the toxicity induced by TNF. To that end we measured apoptosis by annexin V staining. We found that TNF induced a great increase in apoptotic cells in Me180 (Fig. 3B), and a similar increase was observed in MAP17-expressing clones (Fig. 3B). Therefore, although MAP17 expression bypasses the TNF-induced G1 arrest, it seems not to alter TNF-induced apoptosis.

Expression of MAP17 Increases Mannose Uptake in Human Cells

It has been described that the microinjection of MAP17 mRNA in *Xenopus* oocytes triggers the activation of the Na/Mannose transporter, increasing the uptake of mannose and glucose [Blasco et al., 2003]. Increased cellular metabolism and resistance to apoptosis are two hallmarks of cell transformation [Plas and Thompson, 2002]. Glucose uptake and increase of glycolysis are needed to support the ATP:ADP ratio of the transformed cells but also to support survival [Warburg, 1931; Vander Heiden et al., 2001; Kondoh et al., 2005]. Cellular glycolysis can be restricted by reducing the concentration of glucose in the medium and therefore the glucose uptake. Severe restrictions of glycolysis results in cell death via apoptosis [Vander Heiden and Thompson, 1999; Vander Heiden et al., 2000].

Since an increase in mannose/glucose can lead to metabolic changes by increasing metabolism and/or glycosilation/mannosilation of proteins that can alter the TNF response, we decided to explore whether mannose/glucose uptake was responsible for TNF resistance.

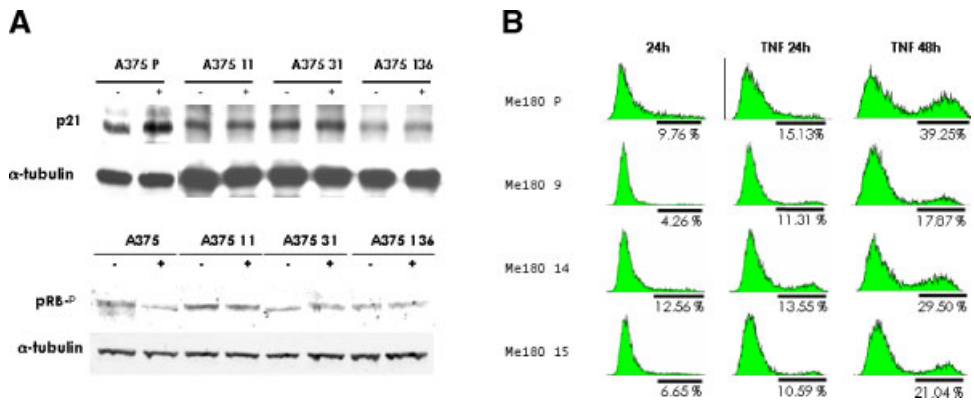

Fig. 3. **A**: Effect of MAP17 ectopic expression in p21waf1 induction and pRb hypophosphorylation after TNFα treatment. Parental or MAP17-expressing cells were seeded and grown until cells reached 70% confluence (approximately). Then, cells were treated with 100 nM TNFα and protein lysates collected after 24 h. Total protein lysates of cells treated (+) or untreated (−) were run in SDS–PAGE, transferred to PVDF membranes and immunostained with anti-p21waf1, anti-α tubulin or anti-phosphorylated retinoblastoma. Data shows that while TNFα induces p21waf1 increase with concomitant pRb dephosphorylation in parental cells, MAP17-expressing clones inhibit the p21waf1 increase maintaining the phosphorylated status of pRb. Parental A375 cells (P) and three representative MAP17 expressing clones (11, 31, 136) are shown. **B**: MAP17 does not inhibit TNFα-induced apoptosis. Parental and MAP17-expressing cells were analyzed for annexin V after 24 and 48 h treatment with 100 nM TNFα. Two populations are separated: living cells and annexin V positive. Percentages correspond to apoptotic Me180 cells. Parental Me180 cell (P); MAP17 expressing clones (9, 14, 15). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Therefore, we first measured whether the expression of MAP17 in human cells induces a similar biological effect. To that end, we measured the uptake of mannose in cells over-expressing MAP17. While in cell lines such as HBL100, ectopic expression of MAP17 causes an increase of radioactive mannose uptake, in A375, T47D of Me180, does not (Fig. 4A). We

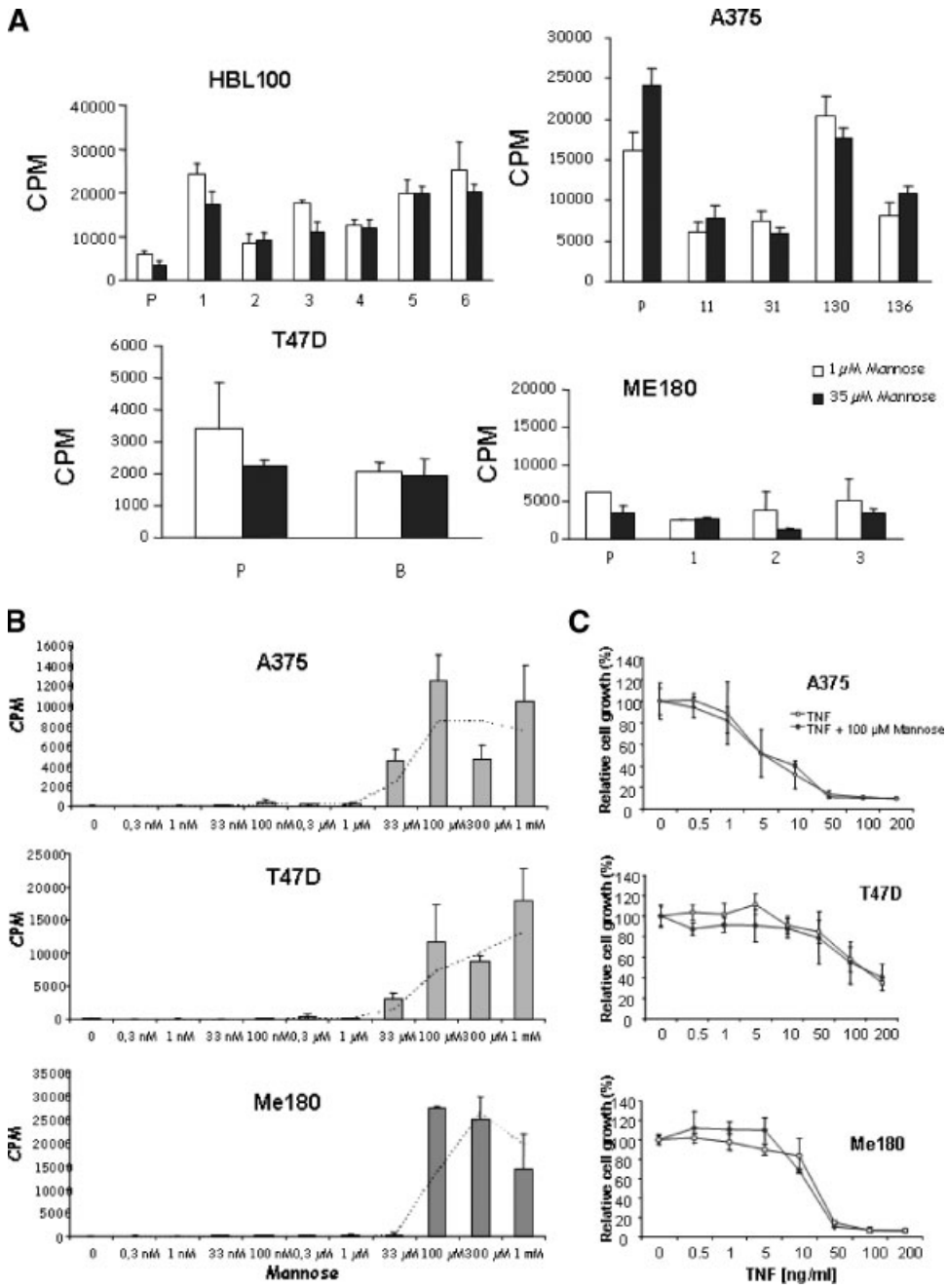

Fig. 4. Expression of MAP17 increases mannose uptake in human cells. **A**: MAP17 increases the uptake of mannose in some cell lines. Parental and MAP17 expressing clones of HBL100, A375, T47D, and Me180 were exposed to D-[2-^{3}H] Mannose for 4 h. Cells were incubated in 1 μM (■) and 35 μM (□) of mannose to stimulate the uptake. See text for details. One representative experiment out of three performed is shown. **B**: Intracellular uptake of mannose depends on mannose levels in the media. Dose-curve of D-[2-^{3}H] Mannose in parental cells. The behavior of the three cell lines is showed with a dotted line. One representative experiment out of three performed is shown. **C**: TNFα effect is independent of the uptake of mannose. Dose-response effect of TNFα (○) or TNFα in 100 μM Mannose (■) in parental cells. Values are expressed as the percentage of cell growth. Untreated control cells represent 100% of cell growth. The data are presented as the mean from triplicate samples; bars, ± SD. One representative experiment out of three performed is shown.

measured mannose uptake at two mannose concentrations (1 and 35 μM) since high concentration of external mannose has been reported to facilitate the uptake. In the cell line HBL100, the MAP17 expressing clones (Fig. 4A) shown a 2- to 5-fold increase of mannose uptake at either 1 or 35 μM mannose over the parental cells carrying empty vector. These results confirm in human cells the biological activity associated to MAP17 in *Xenopus* oocytes reported by Blasco et al. [2003]. However, this is not an universal phenomena since in other cell lines tested (A375, T47D, or Me180), MAP17 ectopic expression does not induce mannose uptake (Fig. 4A). It is possible that this biological activity depends upon the Na/mannose transporter or other related proteins which are present only in certain cell lines.

This precludes the possibility that this internal increase of mannose may be responsible for TNFα resistance. However, to conclude this point, we increased mannose levels exogenously in cells. In a dose-response curve of mannose we observed that internal mannose increases once the mannose reaches levels higher than 30 μM in the medium (Fig. 4B) reaching a plateau up to 1 mM. Then we treated TNF-sensitive cells in the presence of 100 μM mannose. In these conditions in which we increased artificially the internal levels of mannose, we observed that TNF sensitivity was unaltered (Fig. 4C). These results fully confirm that the bypass of TNF induced by MAP17 is independent of the uptake of mannose.

Expression of MAP17 in Human Tumor Cell Lines Does not Correlate With Resistance to TNF

It has been described that around 40% of tumor cell lines are sensitive to TNF [Goeddel et al., 1986]. Therefore, we have analyzed the presence of MAP17 in a panel of tumor cell lines and correlated to TNF resistance. We extracted mRNA from exponentially growing cells and analyzed MAP17 mRNA presence by specific RT-PCR (Table I). Non-tumoral cells used as control (IMR90, WI38, HaCat, and HMVEC) do not express MAP17. Among tumoral cells, 7 out of 28 (25%) express MAP17 mRNA. Next, we analyzed the response of the panel of cells to TNF treatment. We subjected all cell lines to increasing doses of TNF, up to 400 ng/ml and calculated the IC50 for growth inhibition in all cell lines. Those in which IC50 was below 400 ng/ml were considered sensitive. On the other hand, cells which did not reached IC50 at maximum TNF concentrations were considered resistant. We observed that the presence of MAP17 mRNA did not correlate with TNF resistance in all cell lines.

TABLE I. Expression of MAP17 in Human Tumor Cell Lines Does not Correlate With Resistance to TNF

Cell line	Origin	TNF	MAP17 mRNA
MDA-MB 468	Breast carcinoma	S	+
	Breast adenocarcinoma	S	+
SKOV3	Ovary adenocarcinoma	R	+
Hela	Cervix carcinoma	R	+
Du145	Prostate carcinoma	R	+
LnCap	Prostate carcinoma	S	+
COLO205	Colon adenocarcinoma	S	+
IMR90	Normal fibroblasts	R	–
WI38	Normal fibroblasts	R	–
HMVEC	Human microvascular endothelial cells	R	–
HACAT	Immortal keratinocytes	R	–
A375	Metastatic melanoma	S	–
T47D	Breast carcinoma	S	–
MCF7	Breast adenocarcinoma	S	–
HS578 Bst	Breast carcinoma	S	–
BT474	Breast carcinoma	S	–
HTB100	Breast carcinoma	R	–
Me180	Cervix carcinoma	S	–
PC3	Prostate carcinoma	R	–
EM2	Leukemia	S	–
HL60	Leukemia	S	–
K562	Leukemia	R	–
MOLT4	Leukemia	S	–
RAJI	Leukemia	R	–
U87MG	Glioblastoma	S	–

Cell lines of different origin were subjected to RT-PCR and IC50 calculated. TNFα sensitivity (S: IC50 < 400 nM) or resistance (R) and MAP17 expression (+) are indicated.

Our data indicates that although MAP17 can bypass the TNF effect, this is dependent on the cell line and not a general phenomena.

DISCUSSION

Tumoral growth is the result of a deregulation of signals that control cellular differentiation, survival and proliferation. Tumoral growth occurs when tissue homeostasis is disrupted by cells that do not respond to physiological signals. Therefore, genes capable of desensitising tumoral cells from physiological signals may provide a selective advantage within the tumoral mass and influence the outcome of the disease. With the aim of identifying genes with these properties we undertook a large scale genetic screen to select genes able to induce a bypass of TNFα-induced arrest. We identified MAP17, a small membrane-associated protein of 17 kDa. MAP17 is a 17 kDa nonglycosylated membrane protein that locates to the plasma membrane and the Golgi apparatus [Blasco et al., 2003]. The protein consists of a sequence signal peptide, two transmembrane regions and a PDZ domain carboxi-terminus [Jaeger et al., 2000]. The protein is conserved with high homology in Zebrafish (not shown), with the transmembrane domain being the more conserved domain, underlining the importance of membrane localization. MAP17 acts as an atypical anchoring site for PDZK1 and interacts with the NaPi-IIa/PDZK1 protein complex in renal proximal tubular cells [Pribanic et al., 2003]. The physiological role of MAP17 in proximal tubules is not known but stimulates specific Na-dependent transport of mannose and glucose in *Xenopus* oocytes [Blasco et al., 2003] and some human cell lines (Fig. 4).

Bypass of the TNF effect elicited by ectopic expression of MAP17 seems to be specific in two different ways. On one hand, only TNF-induced G1 arrest seems to be inhibited by uncoupling p21waf1 induction. TNF-induced apoptosis seems not to be altered. On the other hand, arrest or toxicity induced by other cytokines such as IFNγ seems not to be altered either. At present we do not know the mechanism responsible for p21waf1 inhibition and therefore for TNF bypass, but this mechanism is not related to mannose uptake, so far the only known physiological activity of MAP17.

In our screening it was our goal to identify genes that might be involved in carcinogenesis by contributing to the bypass of the immunogenic response. MAP17 has been previously found to be associated with carcinomas [Kocher et al., 1995]. Although MAP17 is not the sole determinant of the TNF response (Table I), it is tempting speculate that MAP17 might contribute to the development of tumors by inactivating physiological responses to maintain tissue homeostasis.

In summary, we have identified the Membrane-Associated Protein 17, MAP17, out of a large scale genetic screen to bypass TNF-induced G1 arrest. MAP17 inhibits TNF-induced growth arrest by uncoupling p21 induction and maintaining the phosphorylated status of the pRb protein.

ACKNOWLEDGMENTS

The authors acknowledge the other members of the Assay Development Group for helpful discussions and critical reading of this manuscript. This work has been funded by Spanish Ministry of Health (FIS-02/0126), Fundacion Mutua Madrileña and Spanish Ministry of Education and Science (SAF2005-00944).

REFERENCES

Aggarwal W. 2000. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-kappaB. Ann Rheum Dis 59:i6–i16.

Baker S, Reddy E. 1996. Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene 12:1–9.

Balkwill F, Naylor M, Malik S. 1990. Tumour necrosis factor as an anticancer agent. Eur J Cancer 26:641–644.

Beutler B, Cerami A. 1988. Cachectin (tumor necrosis factor): A macrophage hormone governing cellular metabolism and inflammatory response. Endocr Rev 9: 57–66.

Blasco T, Aramayona J, Alcalde A, Catalan J, Sarasa M, Sorribas V. 2003. Rat kidney MAP17 induces cotransport of Na-mannose and Na-glucose in Xenopus laevis oocytes. Am J Physiol Renal Physiol 285:F799–F810.

Burow M, Weldon C, Collins-Burow B, Ramsey N, McKee A, Klippel A, McLachlan J, Clejan S, Beckman B. 2000. Cross-talk between phosphatidylinositol 3-kinase and sphingomyelinase pathways as a mechanism for cell survival/death decisions. J Biol Chem 275:9628–9635.

Carnero A, Hudson J, Hannon G, Beach D. 2000. Loss-of-function genetics in mammalian cells: The p53 tumor suppressor model. Nucleic Acids Res 28:2234–2241.

Carswell E, Old L, Kassel R, Green S, Fiore N, Williamson B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72:3666–3670.

Chang M, Won S, Yang B, Jan M, Liu H. 1999. Selective activation of Ha-ras(val12) oncogene increases susceptibility of NIH/3T3 cells to TNF-alpha. Exp Cell Res 248:589–598.

Chen G, Goeddel D. 2002. TNF-R1 signaling: A beautiful pathway. Science 296:1634–1635.

Feinman R, Henriksen-DeStefano D, Tsujimoto M, Vilcek J. 1987. Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. J Immunol 138:635–640.

Fernandez A, Marin M, McDonnell T, Ananthaswamy H. 1994. Differential sensitivity of normal and Ha-ras-transformed C3H mouse embryo fibroblasts to tumor necrosis factor: Induction of bcl-2, c-myc, and manganese superoxide dismutase in resistant cells. Oncogene 9:2009–2017.

Goeddel D, Aggarwal B, Gray P, Leung D, Nedwin G, Palladino M, Patton J, Pennica D, Shepard H, Sugarman B. 1986. Tumor necrosis factors: Gene structure and biological activities. Cold Spring Harb Symp Quant Biol 51:597–609.

Hannon G, Sun P, Carnero A, Xie L, Maestro R, Conklin D, Beach D. 1999. MaRX: An approach to genetics in mammalian cells. Science 283:1129–1130.

Haranaka K, Carswell E, Williamson B, Prendergast J, Satomi N, Old L. 1986. Purification, characterization, and antitumor activity of nonrecombinant mouse tumor necrosis factor. Proc Natl Acad Sci USA 83:3949–3953.

Harvat B, Jetten A. 1996. Gamma-interferon induces an irreversible growth arrest in mid-G1 in mammary epithelial cells which correlates with a block in hyperphosphorylation of retionablastoma. Cell Growth Differ 7:289–300.

Hieber U, Heim M. 1994. Tumor necrosis factor for the treatment of malignancies. Oncology 51:142–153.

Jaeger C, Schaefer B, Wallich R, Kramer M. 2000. The membrane-associated protein pKe#192/MAP17 in human keratinocytes. J Invest Dermatol 115:375–380.

Jeoung D, Tang B, Sonenberg M. 1995. Effects of tumor necrosis factor-alpha on antimitogenicity and cell cycle-related proteins in MCF-7 cells. J Biol Chem 270:18367–18373.

Kocher O, Cheresh P, Brown L, Lee S. 1995. Identification of a novel gene, selectively up-regulated in human carcinomas, using the differential display technique. Clin Cancer Res 1:1209–1215.

Kondoh H, Lleonart ME, Gil J, Wang J, Degan P, Peters G, Martinez D, Carnero A, Beach D. 2005. Glycolytic enzymes can modulate cellular life span. Cancer Res 65(1):177–185.

MacEwan D. 2002. TNF receptor subtype signalling: Differences and cellular consequences. Cell Signal 14:477–492.

Merli M, Benassi M, Garnberi G, Ragazzini P, Sollazzo M, Molendini L, Magagnoli G, Ferrari C, Maltarello M, Picci P. 1999. Expression of G1 phase regulators in MG-63 osteosarcoma cell line. Int J Oncol 14(6):1117–1121.

Moneo V, Guijarro M, Link W, Carnero A. 2003. Overexpression of cyclin D1 inhibits TNF-induced growth arrest. J Cell Biochem 89:484–499.

Ozes O, Mayo L, Gustin J, Pfeffer S, Pfeffer L, Donner D. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401: 82–85.

Plas DR, Thompson CB. 2002. Cell metabolism in the regulation of programmed cell death. Trends Endocrinol Metab 13:74–78.

Pribanic S, Gisler S, Bacic D, Madjdpour C, Hernando N, Sorribas V, Gantenbein A, Biber J, Murer H. 2003. Interactions of MAP17 with the NaPi-IIa/PDZK1 protein complex in renal proximal tubular cells. Am J Physiol Renal Physiol 285:F784–F791.

R Development Core Team. 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter M. 1998. Apoptosis signaling by death receptors. Eur J Biochem 254:439–459.

Seliger B, Killian M, Pfizenmaier K. 1988. Distinct mechanisms of interferon-gamma and tumor necrosis factor-alpha action in oncogene-transformed mouse fibroblasts. J Cell Biochem 38:205–212.

Shalaby M, Aggarwal B, Rinderknecht E, Svedersky L, Finkle B, Palladino M. 1985. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. J Immunol 135: 2069–2073.

Shiohara M, Gombart A, Berman J, Koike K, Komiyama A, Koeffler H. 1997. Cytostatic effect of TNFalpha on cancer cells is indjependent of p21WAF1. Oncogene 15:1605–1609.

Sugarman B, Aggarwal B, Hass P, Figari I, Jr., Palladino MA, Shepard H. 1985. Recombinant human tumor necrosis factor-alpha: Effects on proliferation of normal and transformed cells in vitro. Science 230:943–945.

Tracey K, Wei H, Manogue K, Fong Y, Hesse D, Nguyen H, Kuo G, Beutler B, Cotran R, Cerami A. 1988. Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation. J Exp Med 167:1211–1227.

Vander Heiden MG, Thompson CB. 1999. Bcl-2 proteins: Regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1(8):E209–E216.

Vander Heiden MG, Chandel NS, Li XX, Schumacker PT, Colombini M, Thompson CB. 2000. Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. Proc Natl Acad Sci USA 97(9):4666–4671.

Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. 2001. Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol 21(17):5899–5912.

Vilcek J, Lee T. 1991. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. J Biol Chem 266:7313–7316.

Wanebo H. 1989. Tumor necrosis factors. Semin Surg Oncol 5:402–413.

Warburg O. 1956. On the origin of cancer cells. Science 123:309–314.