

Use of a Cloned Multidrug Resistance Gene for Coamplification and Overproduction of Major Excreted Protein, a Transformation-Regulated Secreted Acid Protease

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Malignantly transformed mouse fibroblasts synthesize and secrete large amounts of major excreted protein (MEP), a 39,000-dalton precursor to an acid protease (cathepsin L). To evaluate the possible role of this protease in the transformed phenotype, we transfected cloned genes for mouse or human MEP into mouse NIH 3T3 cells with an expression vector for the dominant, selectable human multidrug resistance (*MDR1*) gene. The cotransfected MEP sequences were efficiently coamplified and transcribed during stepwise selection for multidrug resistance in colchicine. The transfected NIH 3T3 cell lines containing amplified MEP sequences synthesized as much MEP as did Kirsten sarcoma virus-transformed NIH 3T3 cells. The MEP synthesized by cells transfected with the cloned mouse and human MEP genes was also secreted. Elevated synthesis and secretion of MEP by NIH 3T3 cells did not change the nontransformed phenotype of these cells.

MEP, the major excreted protein of transformed mouse cells (10), is the precursor to an acid protease which recently has been identified as cathepsin L (7, 8, 8a, 17, 18). The 39-kilodalton (kDa) mouse MEP is synthesized and secreted in large quantities by malignantly transformed NIH 3T3 cells (9-12) and by phorbol myristate acetate- or platelet-derived growth factor-treated cells (12, 24). Within these cells, the 39-kDa precursor protein is processed to two lower-molecular-mass forms, 29 and 20 kDa, which represent the active forms of the protease in lysosomes (9).

To study the possible function of MEP in growth control and/or transformation of animal cells, it was found necessary to overproduce MEP in otherwise normal cells. We used the human gene (*MDR1*) for multidrug resistance (MDR) as a dominant marker for cotransfection and amplification of the cloned mouse and human MEP genes.

The *MDR1* gene is overexpressed and frequently amplified in various rodent and human cell lines which express simultaneous resistance to drugs such as colchicine, vinblastine, adriamycin, and actinomycin D (6, 22, 25). Resistance is due to the action of the *MDR1* gene product, a 170-kDa, energy-dependent drug efflux pump (22) termed P-glycoprotein (13). A retroviral expression vector containing a full-length cDNA clone of the human *MDR1* gene (pHaMDR1) has been shown to confer MDR when transfected into NIH 3T3 cells (32). We demonstrate here that pHaMDR1 can be used as a dominant selectable marker to cotransfect the MEP gene into NIH 3T3 cells. By selection of transfected cells with increasingly higher concentrations of drug, the *MDR1* DNA and cotransfected MEP sequences are amplified, and MEP is overexpressed.

By amplifying the MEP gene with the MDR selection system, we found it possible to achieve levels of expression

and secretion of MEP comparable to those found in cells malignantly transformed by Kirsten sarcoma virus (KNIH cells). These high levels of MEP had no effect on cell morphology, growth rate, growth in soft agar, growth in low serum, or tumorigenicity in nude mice, suggesting that overexpression and secretion of MEP are not sufficient to mimic any of these aspects of the transformed phenotype.

MATERIALS AND METHODS

Cells. NIH 3T3 cells and KNIH cells were kindly provided by C. Scher (University of Pennsylvania School of Medicine). All cell lines were maintained in Dulbecco modified Eagle medium containing 10% calf serum (Colorado Serum Co.), 5 mM glutamine, 50 U of penicillin per ml, and 50 µg of streptomycin per ml. Colchicine was obtained from Sigma Chemical Co. and diluted from dimethyl sulfoxide stock solutions of 10 mg/ml to appropriate concentrations in complete medium.

Plasmids. pHaMDR1 (previously designated pHaMDR [32]) was constructed as follows. A 4,380-base-pair *SacI-EcoRI* fragment of a human *MDR1* cDNA (32) was modified at its 5' end with a *SacII* linker and at its 3' end with an *XhoI* linker. This fragment was inserted into the *SacII-XhoI* site of the eucaryotic expression vector pCO1 (provided by D. Lowy) to yield pHaMDR1, with the human *MDR1* cDNA sequences inserted between the two long terminal repeats of Harvey murine sarcoma virus. pcosMEP5 is a genomic clone of the mouse MEP gene in the cosmid vector pSV13 (19, 30). pHu16 is a cDNA clone of the human MEP sequences in the Okayama-Berg expression vector (8a, 21). DNAs for transfection were isolated from *Escherichia coli* by standard procedures and purified by two consecutive centrifugations to equilibrium in cesium chloride-ethidium bromide gradients.

Cell transfection and colchicine selection. NIH 3T3 cells were transfected by the calcium phosphate coprecipitation method as described previously (26) with pHaMDR1+pcosMEP5, pHaMDR1+pHu16, or pHaMDR1 only. Control cells received no DNA. Cells were washed after 16 h, and 24 h later they were trypsinized and replated in medium con-

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taining 60 ng of colchicine per ml. After 10 days in selective medium, colonies were pooled into either 40 or 80 ng of colchicine per ml and allowed to grow for at least 10 days. At the same time, individual colonies were picked and grown as clones in 80 ng of colchicine per ml. Both the pooled populations and clones were selected in approximately two-fold increments of colchicine concentration up to 1.5 $\mu\text{g/ml}$ (populations) or 1 $\mu\text{g/ml}$ (clones). At each stage of the selection, cells were allowed to grow in the appropriate drug concentration for at least 5 to 10 days before being plated at the next higher step.

Labeling and immunoprecipitation. Transfected cells were labeled with 50 μCi of [^{35}S]methionine (Amersham Corp.) per ml in Dulbecco-Vogt medium lacking methionine (National Institutes of Health Media Unit) and supplemented with 5 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Labeling was done for 3 h, and labeled culture medium and cell lysates were collected as detailed previously (9). Immunoprecipitations were performed as described previously with either a rabbit anti-mouse MEP antibody to precipitate mouse MEP (11) or a rabbit anti-cathepsin L antibody (a gift from R. W. Mason) to precipitate human MEP. For immunoprecipitations of mouse MEP, 5×10^5 trichloroacetic acid-precipitable counts were used; for immunoprecipitations of human MEP, 5×10^6 cpm were used. Immunoprecipitates were run on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel as described by Laemmli (15). The gel was treated with 2,5-diphenyloxazole in dimethyl sulfoxide (2), and fluorography was performed at -70°C .

DNA isolation and Southern analysis. Genomic DNA was isolated from transfected or control cells essentially as described previously (26). After brief sonication, DNA was digested to completion with *EcoRI*, separated on an 0.8% agarose gel, and transferred to nitrocellulose by the method of Southern (27). Hybridizations were performed at 42°C in 50% formamide- $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0])- $10\times$ Denhardt solution ($1\times$ Denhardt solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll)-50 mM Tris (pH 7.5)-0.1% SDS, 100 μg of single-stranded DNA per ml, and 10^7 dpm of the appropriate probe. Washes were done first with $2\times$ SSC-0.2% SDS at 60°C and then with $0.1\times$ SSC-0.2% SDS at 60°C . The probe for MDR-specific sequences was an internal 3.4-kilobase *EcoRI* fragment isolated from pHaMDR1 and labeled with ^{32}P by nick translation (Lofstrand Labs). The mouse MEP probe was an *EcoRI-PstI* fragment derived from the 3' end of the MEP-coding region of pcosMEP5 (30) and contained some downstream noncoding sequences as well. The MEP probe was labeled with ^{32}P by the random oligonucleotide primer method (Lofstrand Labs). Washed filters were exposed to X-Omat AR film (Eastman Kodak Co.) at -70°C with an intensifying screen.

RNA isolation and RNase protection. Total cell RNA was isolated from transfectants by the guanidine thiocyanate method of Chirgwin et al. (5). For RNase protection analysis, antisense transcripts were synthesized with, as a template, a 110-base-pair *Sau3AI* fragment from a mouse MEP cDNA clone (nucleotides 122 to 232 on the cDNA map [31]) inserted into pGEM-3 (Promega Biotec); the plasmid was linearized at the *PvuII* site of the pGEM-3 vector. The resulting probe, synthesized from the SP6 promoter of pGEM-3, contained 110 nucleotides complementary to mouse MEP mRNA. The RNase protection assays were performed as described by Melton et al. (20) with 10 μg of total cell mRNA and 1.5×10^5 to 3×10^5 cpm of antisense

probe. Final reaction products were resolved on a 6% polyacrylamide gel containing 8 M urea and exposed to X-Omat AR film at -70°C with an intensifying screen.

RESULTS

Cotransfection and coamplification of MDR and MEP. Plasmid pHaMDR1 contains a full-length cDNA clone of the human *MDR1* gene inserted between two Harvey murine sarcoma virus long terminal repeats. This plasmid was previously shown to confer MDR when transfected into NIH 3T3 cells (32).

The cosmid pcosMEP5 is a genomic clone of the mouse MEP (mouse cathepsin L) gene, while pHu16 is a full-length cDNA clone of the human MEP (human cathepsin L) homolog in the Okayama-Berg expression vector. The mouse genomic clone expresses the full 39-kDa precursor form of MEP when transfected into CV-1 or A431 cells (30). This protein is secreted and processed normally by the transfected cells and is enzymatically active. Likewise, the human cDNA clone expresses in transfected NIH 3T3 cells a 42-kDa protein which comigrates on SDS-polyacrylamide gels with the human homolog of MEP from A431 cells (data not shown).

We used *MDR1* as a selectable marker in cotransfections with either the mouse or human MEP clone. NIH 3T3 cells were transfected with pHaMDR1+pcosMEP5 DNA, with pHaMDR1+pHu16 DNA, or with pHaMDR1 DNA only as described in Materials and Methods. Transfected cells were initially selected in medium containing 60 ng of colchicine per ml, a drug concentration at which mock-transfected cells yielded no background colonies. Transfections with plasmid pHaMDR1 resulted in colchicine-resistant colonies at a frequency of 5×10^{-4} to 2×10^{-3} . After 10 days in selective medium, 100 to 300 colchicine-resistant colonies were pooled into either 40 or 80 ng of colchicine per ml and allowed to grow in culture for at least 10 days. For some of the experiments described below, individual colonies of transfected cells were picked and grown initially in 80 ng of colchicine per ml. To amplify the transfected *MDR1* sequences, we selected cells with increasing concentrations of colchicine in the culture medium (see Materials and Methods).

To analyze MEP expression in transfected cells, pooled colonies growing in the presence of 40, 160, and 640 ng and 1 μg of colchicine per ml were labeled metabolically with [^{35}S]methionine, and immunoprecipitations were performed on cell lysates and culture medium. The results (Fig. 1) indicated that colchicine-resistant cells also expressed the cotransfected MEP sequences. (In the case of the pHaMDR1+pcosMEP5 transfections, protein made from the MEP cosmid comigrated with the low level of endogenous MEP produced by NIH 3T3 cells.) Furthermore, as colchicine resistance increased, MEP synthesis also was amplified. MEP synthesized by transfected cells was compared with MEP produced by KNIH cells, derivatives of NIH 3T3 cells transformed by Kirsten sarcoma virus (Fig. 1, lane 1). KNIH cells synthesize about 50-fold more MEP than do NIH 3T3 cells and secrete 50 to 60% of the 39-kDa form of this protein into the culture medium. In the presence of 1 μg of colchicine per ml, the pHaMDR1+pcosMEP5 cell line synthesized and secreted at least as much MEP as did KNIH cells.

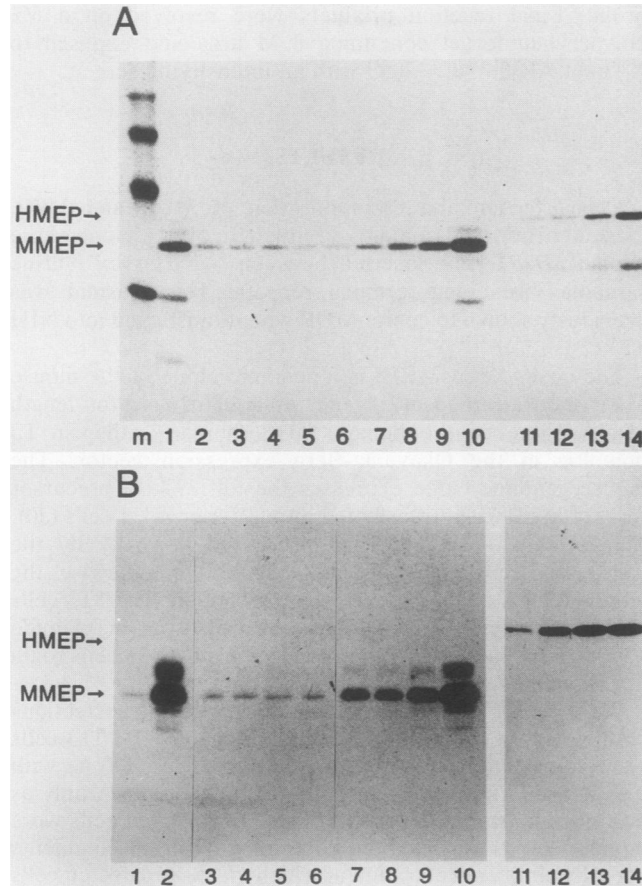


FIG. 1. MEP in colchicine-selected transfections. Populations of transfectants containing pHaMDR1 DNA alone, pHaMDR1+pcosMEP5 DNA, or pHaMDR1+pHu16 DNA were labeled with [³⁵S]methionine for 3 h. Cell lysates (A) or culture supernatants (B) were then immunoprecipitated with anti-MEP antibody as described in Materials and Methods. Pictured are fluorograms of the resulting SDS-polyacrylamide gels. (A) Immunoprecipitation of cell lysates of KNIH cells (lane 1); NIH 3T3 cells (lane 2); pHaMDR1 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml (lanes 3 to 6, respectively); pHaMDR1+pcosMEP5 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml (lanes 7 to 10, respectively); and pHaMDR1+pHu16 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml (lanes 11 to 14, respectively). For lanes 1 to 10, precipitations were performed with α -mouse MEP antibody and 5×10^5 trichloroacetic acid-precipitable counts of lysate; for lanes 11 to 14, precipitations were performed with α -human cathepsin L antibody and 5×10^6 cpm of lysate. m, Protein molecular mass standards of 68, 43, 25.7, and 18.4 kDa, from top to bottom. (B) Immunoprecipitation of culture medium of labeled cells. Lanes 1 and 2 are NIH 3T3 and KNIH cells, respectively. For all other lanes, antibodies and counts are as described for panel A.

Interestingly, secretion of both the transfected mouse and transfected human proteins also increased with higher colchicine resistance (Fig. 1B), suggesting that secretion of MEP by growth-stimulated NIH 3T3 cells or by KNIH cells could simply be a result of overexpression of the lysosomal protein rather than a secondary effect of growth stimulation or transformation.

Amplification of MEP sequences. To study the mechanism of increased drug resistance and increased MEP synthesis in transfected cells, we analyzed RNA and DNA from these cell lines. Steady-state MEP mRNA levels in transfected populations were measured by RNase protection analysis

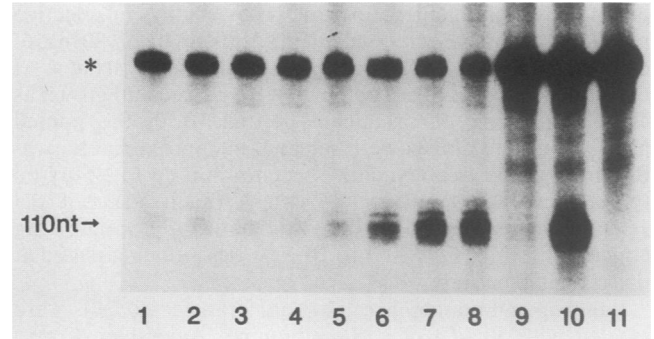


FIG. 2. RNase protection analysis. Total cell RNA was isolated from populations of pHaMDR1 or pHaMDR1+pcosMEP5 transfectants by the method of Chirgwin et al. (5). RNase protection was performed with 10 μ g of RNA and an antisense RNA probe which protects 110 nucleotides of internal MEP coding sequences within both the transfected MEP mRNA and the endogenous mouse MEP mRNA of NIH 3T3 cells (see Materials and Methods). Illustrated is an autoradiogram of the RNase reaction products separated on a 6% polyacrylamide gel. Lanes: 1 to 4, RNA from pHaMDR1 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml, respectively; 5 to 8, RNA from pHaMDR1+pcosMEP5 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml, respectively; 9, RNA from NIH 3T3 cells; 10, RNA from KNIH cells; 11, no RNA. The position of 110 nucleotide (110nt) band is indicated on the left. The asterisk represents undigested probe (for lanes 9 to 11, probes for both mouse MEP and human MEP [data not shown] were used, accounting for the more intense band of undigested probe; the human MEP probe does not cross-react with mouse MEP mRNA in NIH 3T3 cells.)

(see Materials and Methods). Figure 2 shows that MEP mRNA increased with increasing colchicine resistance in pHaMDR1+pcosMEP5 transfectants (Fig. 2, lanes 5 to 8). This increase paralleled the protein enrichments during selection. MEP mRNA in control pHaMDR1 transfectants (Fig. 2, lanes 1 to 4) remained constant at the low endogenous levels normally seen in NIH 3T3 cells (lane 9). Similar results were obtained for the pHaMDR1+pHu16 transfectants (data not shown).

To determine whether increased MDR and MEP expression was accompanied by amplification of the respective DNAs, we picked individual clones from the initial transfections with pHaMDR1, pHaMDR1+pcosMEP5, or pHaMDR1+pHu16. Each clone was subsequently grown in stepwise increments of colchicine as described above, and genomic DNA was isolated from cells growing in the presence of 80, 160, and 640 ng and 1 μ g of colchicine per ml. DNA was digested with *EcoRI*, separated on an 0.8% agarose gel, and transferred to nitrocellulose filters. Filters were hybridized with probes specific for either *MDR1* or MEP. The results for one clone from the mouse MEP transfection are shown in Fig. 3. *MDR1* and MEP sequences were amplified in parallel in the transfectants as colchicine resistance increased.

Effect of MEP overexpression. MEP expression and secretion are stimulated in transfected NIH 3T3 cells and in cells treated with tumor promoters or growth factors. MEP might therefore be a primary stimulator of cell growth and/or a primary cause of the transformed state. To investigate this possibility, we analyzed the phenotypes of populations of transfected cells which overexpressed MEP in the absence of any other transformation or growth stimulus.

pHaMDR1+pcosMEP5 cells resistant to 1 μ g of colchicine per ml produced almost 20-fold more MEP than did

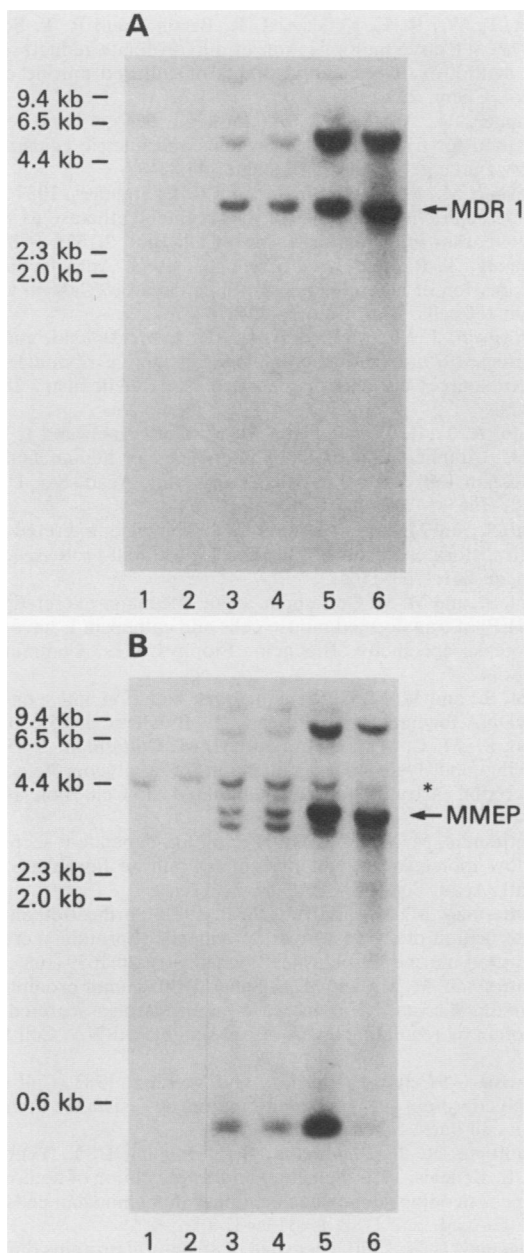


FIG. 3. Southern analysis of genomic DNAs. Genomic DNA was isolated from a cloned cell line of pHaMDR1+pcosMEP5 transfectants growing in the presence of 80, 160, and 640 ng and 1 μ g of colchicine per ml. DNA was digested with *Eco*RI, separated on an 0.8% agarose gel, and transferred to nitrocellulose. Shown are the autoradiograms of the resulting Southern analyses (see Materials and Methods). (A) A filter was hybridized with a 3.4-kb *Eco*RI fragment isolated from the MDR coding region of pHaMDR1. (B) A duplicate filter was hybridized with a mouse MEP-specific probe which detects an MEP-pSV13 junction fragment from the transfected cosmid pcosMEP5A (MMPEP). The probe also hybridized with the single-copy endogenous mouse MEP gene, indicated by the asterisk. Lanes: 1 and 2, NIH 3T3 and KNIH cells, respectively; 3 to 6, pHaMDR1+pcosMEP5 clone at 80, 160, and 640 ng and 1 μ g of colchicine per ml, respectively. Positions of DNA standards are indicated to the left of each panel. Multiple bands are due to multiple integration sites of the transfected DNA.

TABLE 1. Transformation assays

Cell line	Colonies in soft agar ^a	Growth in 0.5% serum (%) ^b	Tumorigenicity ^c
pHaMDR1+pcosMEP5	0	0	-
pHaMDR1+pHu16	0	0	ND
pHaMDR1	0	0	-
NIH 3T3	0	0	-
KNIH	250	82	+
K-MDR	155	59	+

^a Duplicate 10-cm dishes were seeded with 600 cells in 0.35% agar onto a feeder layer of 0.5% agar. Values are the averages of the number of colonies formed after 10 days.

^b Growth in complete medium containing 0.5% calf serum was determined as the plating efficiency relative to 100% efficiency in 10% calf serum. Values are the averages of duplicate assays.

^c Nude mice were injected subcutaneously with transfected or control populations of cells and monitored for tumor formation. Results for each cell line are presented as tumors detected (+) or not detected (-) 19 days after injection of 10^6 cells into each of three mice. Delayed tumor growth (greater than 3 weeks) was detected in some of the mice injected with 1×10^6 or 5×10^6 cells of the pHaMDR1 or NIH 3T3 populations, but no tumors were seen with the NIH 3T3 cells transfected with pHaMDR1+pcosMEP5 up to 5 weeks after injection of 5×10^6 cells. ND, Not done.

pHaMDR1 transfectants with the same drug resistance, a level of MEP nearly comparable to that in transformed KNIH cells. pHaMDR1+pHu16 cells grown in the presence of 1 μ g of colchicine per ml expressed about 10-fold more MEP than did the corresponding transfectants grown at 40 ng of colchicine per ml. Populations of these MEP cells were analyzed for cell morphology, growth rate, growth in soft agar, growth in low serum, and tumorigenicity relative to control cells.

As a control for the effect of colchicine or *MDR1* expression in these experiments, KNIH cells were transfected with the *MDR1* gene and selected stepwise with colchicine up to 1 μ g/ml as described above. The resulting cells were designated K-MDR cells. Both KNIH and K-MDR cells exhibited a more rounded shape than did NIH 3T3 cells and were not contact inhibited in culture. They grew about twice as fast as NIH 3T3 cells, readily formed colonies in soft agar, were able to grow in low serum, and caused tumors when injected into nude mice.

The transfected NIH 3T3 cells producing similar amounts of MEP as KNIH cells appeared morphologically normal and grew at a rate similar to that of control cells transfected with pHaMDR1 (data not shown).

The results of three assays of malignant transformation are summarized in Table 1. Growth in soft agar was used to determine whether MEP overexpression could confer anchorage independence on NIH 3T3 cells. Whereas KNIH and K-MDR cells formed colonies in soft agar after 7 to 10 days, we saw no colonies with the *MDR1*-MEP cell lines or with the control NIH 3T3 or *MDR1* cells. Growth in low serum was assayed by plating cells in medium containing 10, 5, 2, 1, 0.2, or 0.1% serum. The relative cloning efficiencies of the cell lines indicated that KNIH and K-MDR cells were able to grow in medium containing only 0.5% serum, while NIH 3T3 cells and all of the transfected populations required 1 to 2% serum. Finally, cells synthesizing and secreting MEP were not tumorigenic in nude mice, even at the highest dose of injected cells (5×10^6 cells per mouse), at least 4 weeks after injection. KNIH and K-MDR cells caused tumors within 1 week at doses that were 5- to 10-fold lower.

DISCUSSION

We now report that the *MDR1* gene is an excellent dominant, selectable marker in NIH 3T3 cells. The pHaMDR1 expression vector has been transfected into a variety of other rodent and human cell lines and confers drug resistance with good efficiency (unpublished results). In addition, the *MDR1* gene has the advantage of being easily amplified in these cell lines, with a variety of drugs as the selective agents. Other such selectable, amplifiable markers include the dihydrofolate reductase gene (1, 28), the *E. coli* gene for asparagine synthetase (3), the adenosine deaminase gene (14), and the ornithine decarboxylase gene (4).

Further, we have used the human *MDR1* gene as a means of cotransfecting the nonselectable MEP gene into NIH 3T3 cells. Probably by virtue of its cointegration along with *MDR1* DNA into the host genome, MEP DNA was also coamplified upon selection with progressively increasing concentrations of colchicine in the culture medium. We were able to attain very high expression of MEP in otherwise normal cells, reaching MEP levels comparable to those seen in transformed KNIH cells.

The overproduction of MEP led to the increased secretion of the protein by the transfected cells, suggesting that the secretion of MEP by KNIH cells is not a secondary effect of the transformed state of those cells but results directly from the presence of large amounts of the lysosomal protein. The effect on the lysosomal or secretory pathway is a specific one, however, since other lysosomal proteins are not coordinately secreted along with MEP (23). This phenomenon is similar to that observed with carboxypeptidase Y in yeast (29). These results suggest that there might be specific, saturable receptors for proteins in the sorting and transport machinery of cells.

Transfected cells which expressed and secreted large amounts of MEP appeared to be phenotypically normal in several transformation assays. When the population of pHaMDR1+pcosMEP5 transfectants growing in the presence of 1 μ g of colchicine per ml was subcloned, 8 of 10 individual clones expressed levels of MEP at least 20-fold higher than those in control cells (unpublished results), suggesting that we should have been able to detect any effects of MEP on transformation in our assays with cell populations. Thus, MEP overexpression and secretion are not sufficient for inducing the transformed state of NIH 3T3 cells. However, MEP might be involved in malignancy in other ways. If it is not a primary agent in transformation or growth control, it might act in coordination with other factors, such as the *c-myc* or adenovirus E1a genes (16), to elicit a growth response.

With the ability to express very high levels of MEP without requiring cell transformation or stimulation by growth factors, we are in a position to understand the possible function of this lysosomal protease in growth control and the transformed phenotype. The MDR system, which appears to serve as a strong dominant marker which can be easily amplified in many cell types, should be useful for these studies and others requiring high-level gene expression in transfected cells.

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