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The Product of the *EMS1* Gene, Amplified and Overexpressed in Human Carcinomas, Is Homologous to a v-*src* Substrate and Is Located in Cell-Substratum Contact Sites

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We have previously identified two genes (*EMS1* and *PRAD1*/cyclin D1) in the chromosome 11q13 region that are frequently coamplified and overexpressed in human breast cancer and in squamous cell carcinomas of the head and neck (E. Schuuring, E. Verhoeven, W. J. Mooi, and R. J. A. M. Michalides, Oncogene 7:355–361, 1992). We now report on the characterization of the 80/85-kDa protein that is encoded by the *EMS1* gene. Amino acid sequence comparison shows a high homology (85%) to a chicken protein that was recently identified as a substrate for the *src* oncogene (H. Wu, A. B. Reynolds, S. B. Kanner, R. R. Vines, and J. T. Parsons, Mol. Cell. Biol. 11:5113–5124, 1991). Immunocytochemistry reveals that in epithelial cells, the human *EMS1* protein is localized mainly in the cytoplasm and, to a very low extent, in protruding leading lamellae of the cell. However, in carcinoma cells that constitutively overexpress the protein as a result of amplification of the *EMS1* gene, the protein, except in cytoplasm, accumulates in the podosome-like adherens junctions associated with the cell-substratum contact sites. The protein was not found in intercellular adherens junctions. Our findings, and the previously reported observations in *src*-transformed chicken embryo fibroblasts, suggest that the *EMS1* protein is involved in regulating the interactions between components of adherens-type junctions. Since amplification of the 11q13 region has been associated with an enhanced invasive potential of these tumors, overexpression and concomitant accumulation of the *EMS1* protein in the cell-substratum contact sites might, therefore, contribute to the invasive potential of these tumor cells.

Amplification of DNA markers within the chromosome 11q13 region (*INT2/FGF3*, *HSTF1/FGF4*, and *BCL1*) occurs in a variety of common human malignant tumors (reviewed in reference 16) and is associated in breast cancer (1, 2, 43, 45) and in squamous cell carcinomas of the head and neck (13) with an unfavorable clinical course of disease. Expression of the two known proto-oncogenes within the 11q13 region, *INT2/FGF3*, and *HSTF1/FGF4*, is only rarely observed in breast tumors with an 11q13 amplification (9, 21, 42). These proto-oncogenes, therefore, are most likely not the major driving force for the amplification unit. We recently identified two candidate genes within the amplified chromosome 11q13 region, *PRAD1*/cyclin D1 and *EMS1* (42). The *PRAD1*/cyclin D1 gene was also independently cloned from a parathyroid adenoma (26) and from a B-cell lymphoma (47) and appeared in those cases overexpressed as the result of a chromosomal translocation (26, 36, 47). In breast carcinomas and in squamous cell carcinomas of the head and neck, the *PRAD1*/cyclin D1 gene is overexpressed as the result of gene amplification (17, 42). The *PRAD1*/cyclin D1 gene appears to be involved in regulation of the cell cycle (19, 24, 26, 50). The other candidate gene within the amplified 11q13 region, *EMS1*, was also amplified and overexpressed in the large majority of tumors with an 11q13 amplification. Moreover, overexpression of *EMS1* and *PRAD1*/cyclin D1 is not restricted to tumors with an 11q13 amplification, indicating that mechanisms other than amplification may also activate these putative oncogenes (42).

Here, we report on the characterization of the product of the *EMS1* gene. The sequence of the protein deduced from an *EMS1* cDNA clone is homologous to the sequence of a v-Src substrate. The human *EMS1* protein is localized in cytoplasm and areas of cell-substratum contacts, in either protruding leading lamellae or podosome-like structures, depending on amplification of the *EMS1* gene.

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MATERIALS AND METHODS

Identification of full-length *EMS1* cDNA clones. A λgt11 cDNA library generated from size-fractionated mRNA (3.0 to 4.0 kb) derived from the human mammary tumor cell line T47D (20; obtained from M. Ligtenberg, Netherlands Cancer Institute, Amsterdam) was screened with the 3′ *EMS1* cDNA probe pU21C8B (42). To identify the longest cDNA, a 5′ extension cloning procedure was used as outlined in Fig. 1a. DNA of 30 positive phage clones was used in a polymerase chain reaction (PCR) with either the 18-mer forward or backward M13/pUC primer (Boehringer, Mannheim, Germany) and a specific primer (primer 1; 5′-GGTTTCAGGAG GATTCCTTAC-3′); the PCR products were size fractionated on agarose gels and hybridized with primer 2 (5′-GTTACACATATAAGGGGAGGG-3′). Phage clone 36 (T47ϕ36) harboring a 2.9-kb insert was subcloned into the *Eco*RI site of pUC9, and the 5′ end was sequenced by using the forward and reverse 17-mer pUC/M13 oligonucleotide sequencing primers (Boehringer). A probe from the 5′ end (0.5-kb *Pvu*II-*Pvu*II fragment) was used to rescreen the pU21C8B positive plaques in the same cDNA library. With DNA isolated from positive plaques, the longest cDNA (T47ϕ52) was subsequently identified with a similar 5′ extension cloning procedure using primer 3 (5′-TGCTTG

a

43 21

E S H HNP P S B X EEE EMS1 cDNA

0 1000 2000 3000 3248 bp

U21C8B

T47φ36

T47φ52

T47f36.52

b

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p80/85   1                                                MTVLLLVVLQ

EMS1     1 MWKASAGHAV SIAQDDAGAD DWETDPDFVN DVSEKEQRWG AKTVQGSGHQ
p80/85  11 MWKATAGHSI AVSQDD.GAD DWETDPDFVN DVSEKEQRWG AKTVKGSGHQ
HS1      1 MWKSVVGHDV SVSVETQG.D DWDTDPDFVN DISEKEQRWG AKTIEGSGRT

EMS1    51 EHINIHKLRE NVFQEHQTLK EKELETGPKA SHGYGGKFGV EQDRMDKSAV
p80/85  60 EHINIHQLRE NVFQEHQTIK EKELETGPKA SHGYGGKFGV EQDRMDKSAV
HS1     50 EHINIHQLRN KVSEEHDVLR KKEMESGPKA SHGYGGRFGV ERDRMDKSAV

EMS1   101 GHEYQSKLSK HCSQVDSVRG FGGKFGVQMD RVDQSAVGFE YQGKTEKHAS
p80/85 110 GHEYQSKLSK HCSQVDSVKG FGGKFGVQTD RVDQSAVGFE YQGKTEKHAS
HS1    100 GHEYVAEVEK HSSQTDAAKG FGGKYGVERD RADKSAVGFD YKGEVEKHTS

EMS1   151 QKDYSSGFGG KYGVQADRVD KSAVGFDYQG KTEKHESQRD YSKGFGGKYG
p80/85 160 QKDYSSGFGG KYGVQADRVD KSAVGFDYQG KTEKHESQKD YSKGFGGKYG
HS1    150 QKDYSRGFGG RYGVEKDKWD KAALGYDYKG ETEKHESQRD YAKGFGGQYG

EMS1   201 IDKDKVDKSA VGFEYQGKTE KHESQKDYVK GFGGKFGVQT DRQDKCALGW
p80/85 210 VDKDKVDKSA VGFEYQGKTE KHESQKDYVK GFGGKFGVQT DRQDKCALGW
HS1    200 IQKDRVDKSA VG........ .......... .......... ..........

EMS1   251 DHQEKLQLHE SQKDYKTGFG GKFGVQSERQ DSAAVGFDYK EKLAKHESQQ
p80/85 260 DHQEKVQLHE SQKDYKSGFG GKFGVQTERQ DPSAVGFDYK EKLAKHESQQ
HS1        .......... .......... .......... .......... ..........

EMS1   301 DYSKGFGGKY GVQKDRMDKN ASTFEDVTQV SSAYQKTVPV EAVTSKTSNI
p80/85 310 DYSKGFGGKY GVQKDRMDKN AATFEDIEKP TSTYQKTKPV ERVANKTSSI
HS1    212 .......... .......... ...FNEMEAP TTAYKKTTPI EAASSGARGL

EMS1   351 RANFENLAKE KEQEDRRKAE AERAQRMAKE RQEQEEA... ..........
p80/85 360 RANLENLAKE KEQEDRRKAE AERAQRMARE KQEQEEA... ..........
HS1    239 KAKFESMAEE K....RKREE EEKAQQVARR QQERKAVTKR SPEAPQPVIA

EMS1   388 .......... RRKLEEQARA KTQTPPVSPA PQPTEERLPS SPVYEDAASF
p80/85 397 .......... RRKLEEQAKA KKQTPPPSPT TQPAEPKTPS SPVYQDAVSY
HS1    285 MEEPAVPAPL PKKISSEAWP PVGTPPSSES EPVRTSREHP VPLLPIRQTL

EMS1   428 KAELSYRGP. ...VSG.... ...TEPEPVY SMEAADYREA SSQQGLAYAT
p80/85 437 DAESAYKNSS TTYSAE.... ...HEPESGY KTTGSDYQEA VSQREAEYEP
HS1    335 PEDNEEPPA. ...LPPRTLE GLQVEEEPVY EA......EP EPEPEPEPEP

EMS1   467 EAVYESAEAP GHYPAEDSTY DEYEN..... .......... ..........
p80/85 480 ETVYEVAGAG DHYQAEENTY DEYEN..... .......... ..........
HS1    375 ENDYEDVEEM DRHEQEDEPE GDYEEVLEPE DSSFSSALAG SSGCPAGAGA

EMS1   492 ...DLGYTAV ALYDYQAAGD DEISFDPDDI ITNIEMIDDG WWRGVCKGRY
p80/85 505 ...ELGITAI ALYDYQAAGD DEISFDPDDI ITNIEMIDDG WWRGVCKGRY
HS1    425 GAVALGISAV ALYDYQGEGS DELSFDPDDV ITDIEMVDEG WWRGRCHGHF

EMS1   539 GLFPANYVEL RQ  550
p80/85 552 GLFPANYVEL RQ  563
HS1    475 GLFPANYVKL LE  487
```

c

1 100 200 300 400 500 550 aa

NH2- -COOH

A B C D

ra14 ra23

FIG. 1. (a) Characterization of *EMS1* transcripts. Schematic representation of the cDNA clones isolated with the 1.35-kb cDNA clone pU21C8B (42) and restriction map of the consensus cDNA sequence of *EMS1* (top). The coding domain is indicated by the bar. The U21 and T47 cDNA clones were derived from the squamous cell carcinoma UMSCC2 and the mammary tumor cell line T47D, respectively. Restriction sites: B, *Bam*HI; E, *Eco*RI; N, *Nco*I; X, *Xba*I; H, *Hin*dIII; P, *Pvu*II; S, *Sma*I. Positions of the oligonucleotide primers 1 to 4 used for 5′ extension PCR cloning are also indicated. (b) Amino acid sequence comparison (in one-letter amino acid code) of the protein encoded by the

GAAAGTTTCGACTG-3') and primer 4 (5'-TTTCCCTCCA TAGCCAATGGG-3'). This PCR fragment (of two independent reactions) was subcloned, and several clones were sequenced. A 420-bp *Nco*I fragment of this PCR product was cloned into the *Nco*I site of clone T47ϕ36, resulting in a 3.2-kb cDNA clone, T47f36.52.

DNA sequencing and analysis. The cDNA *EMS1* clones were subcloned in the plasmid vector pUC9 and sequenced as previously described (42). Briefly, the double-stranded DNA of the clones (U21C8B, T47ϕ36, and the 500-bp 5' ends of clones T47ϕ52 and T47f36.52) and subclones was sequenced by the dideoxy-chain termination method (38), using Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) with the forward and reverse 17-mer pUC/M13 oligonucleotide sequencing primers and with four sequence-specific synthetic primers (primers 1 to 4; Fig. 1a). The synthetic primers were synthesized on a Cyclone Plus synthesizer (Biosearch), extracted with diethyl ether, precipitated with 0.1 volume of sodium acetate and 2 volumes of ethanol, and dissolved in 10 mM Tris-HCl (pH 7.5)–0.1 mM EDTA.

PCR and plasmid/phage cloning procedures were performed according to standard methods (37); T4 ligase and calf intestinal alkaline phosphatase were purchased from Boehringer; *Taq* polymerase was purchased from Perkin-Elmer-Cetus (Berkeley, Calif.).

Assembly of DNA sequences and searches for open reading frames and for potential functional signatures in the *EMS1* protein were performed with the Genetics Computer Group sequence analysis software package (8) and will be described in detail elsewhere (41). The GenBank DNA data bank (release 66.0) and the National Biomedical Research Foundation (NBRF; release 25.0) and SWISS-PROT (release 16.0) protein data banks were searched for homologies to the cDNA sequence. The program Fasta, based on the algorithm of Pearson and Lipman (8, 31), was used for nucleic acid homologies with word size equal to 6 and for protein sequence homologies with word size equal to 2.

Cell culture. Cell lines UMSCC1, UMSCC2, UMSCC8, and UMSCC22B, derived from squamous cell carcinomas of the head and neck region of the upper digestive tract, were provided by T. Carey (Department of Otolaryngology, University of Michigan, Ann Arbor). The normal mammary epithelial cell line HBL100 and the breast cancer cell lines T47D and MDA-MB-134-VI were provided by the American Type Culture Collection. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) (all purchased from GIBCO BRL, Grand Island, N.Y.). Cells were passaged twice weekly with 0.25% (wt/vol) trypsin and 0.02% (wt/vol) EDTA. All cell lines were tested for mycoplasma contamination routinely every month.

The level of amplification of the DNA markers within the chromosome 11q13 region was determined with Southern blot analysis as described previously (42). Double-labeled fluorescence in situ hybridization with probes from the chromosome 11q13 markers in combination with a chromosome painting using a chromosome 11-specific library was used to distinguish specific 11q13 amplification from polyploidy as described recently (33). In this study, three cell lines with specific 11q13 homogeneous staining regions (UMSCC2, UMSCC22B, and MDA-MB-134-IV) and four cell lines with single copies (UMSCC1, UMSCC8, HBL100, and T47D) were used. In all of these cell lines, the level of *EMS1* overexpression is correlated with 11q13 amplification (42).

Generation of antisera directed to the *EMS1* protein. For the production of *Escherichia coli*-expressed *EMS1*-containing fusion proteins, two fragments comprising different domains in the *EMS1* protein (ra14 and ra23; Fig. 1c) were subcloned each into two bacterial expression vectors, pUEX and pATH (3, 11). The cloning sites were sequenced to confirm in-frame translation. Preimmune sera of rabbits used for immunization were tested for lack of reactivity in immunoblotting with preparations of fusion proteins. To generate antibodies against the *EMS1* protein, the *E. coli*-expressed β-galactosidase–*EMS1* fusion proteins (ra14 and ra23) were purified as inclusion bodies and used for immunization of rabbits according to a standard protocol (11). The *E. coli*-expressed TrpE-*EMS1* fusion proteins were purified as described for the β-galactosidase–*EMS1* fusion proteins (11) and used as antigens to test the reactivity of the antisera obtained from the rabbits immunized with either the ra14 or ra23 β-galactosidase–*EMS1* fusion protein (e.g., Fig. 3a).

Antibodies directed against two separate domains of the *EMS1* protein were affinity purified on the corresponding and preabsorbed on the noncorresponding β-galactosidase–*EMS1* fusion protein coupled covalently to a glutaraldehyde-activated affinity adsorbent (Boehringer). With this procedure, antibodies reactive with β-galactosidase and (contaminating) bacterial proteins present in the inclusion bodies were removed from the affinity-purified fraction.

Immunoprecipitation and Western immunoblot analysis. Cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in lysis buffer (0.2×10^7 to 1.0×10^7 cells per ml) containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 10 mM EDTA, 100 mM NaCl, 0.02% phenylmethylsulfonyl fluoride, and 0.002% aprotinin. An aliquot of the lysate representing approximately 10^6 cells was boiled for 2.5 min at 100°C in the presence of sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 10% β-mercaptoethanol, 60 mM Tris buffer [pH 6.8], 0.001% bromophenol blue), and the dissolved proteins were subjected to SDS–8% polyacrylamide gel electrophoresis (PAGE) as described previously (15). The proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, Mass.) and stained with Ponceau S; after destaining and blocking with 1% bovine serum albumin–0.05% Tween 20–PBS, the membranes were incubated with

EMS1 gene with the chicken p80/p85 protein (48) (GenBank accession number M73705) and with a protein predicted from the human *HS1* cDNA (14) (GenBank accession number X16663). (c) Structural features of the *EMS1* protein predicted from the nucleotide sequence. Domain A, six tandem repeats of 37 amino acid residues and a partial repeat of 23 residues (residues 81 to 325). Each repeat motif in domain A is indicated with an arrow. Domain B, a predicted α-helical region (residues 355 to 395). Domain C, region rich in threonines, prolines, and serines (residues 401 to 447). Domain D, SH3 motif (residues 498 to 546). Solid bars represent the region of the *EMS1* proteins ra14 (residues 100 to 258) and ra23 (residues 323 to 393) used to construct fusion proteins to generate rabbit antisera. The vertical lines with symbols over the diagram indicate positions of the potential phosphorylation sites for casein kinase II (●), for protein kinase C (○) (as determined by using the program UWGCG/PROSITE 8.0) and for $p34^{cdc2}$ kinase (▽; P/A-D/V/L-S/T-X-basic [18, 25]). Vertical lines without symbols indicate positions of the tyrosine residues.

either ra14 or ra23 total antiserum (dilution of 1:300) or with ra14 or ra23 preabsorbed and affinity-purified antibodies (dilution of 1:100). The reactive proteins were visualized with an anti-rabbit alkaline phosphatase (dilution of 1:000) with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium as instructed by the manufacturer (Promega, Madison, Wis.). Normal rabbit preimmune serum (dilution of 1:300) served as a negative control (data not shown). A Rainbow molecular weight marker (Amersham, Amersham, United Kingdom) was used on each gel.

For the immunoprecipitations, 2 μl of antiserum or normal serum was incubated with 100 μl of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) in 500 μl of lysis buffer for 1 h at 12°C. Three to five milligrams of protein lysate was subsequently precleared two times by incubation for 30 min at 12°C with protein A beads (Repligen, Cambridge Mass.) precoated with normal rabbit serum (Dako, Glostrup, Denmark); the specific protein was absorbed from the lysate by incubation with beads coated with either ra14 or ra23 antiserum for 30 min at 12°C. The beads were washed four times in lysis buffer and once in lysis buffer without Nonidet P-40. The immunoprecipitates were incubated for 60 s at 100°C in the presence of sample buffer and subjected on SDS-PAGE.

Immunofluorescence. For immunostaining, cells were grown in multichamber slides (purchased from GIBCO BRL). Cells, either untreated or cultured in the presence of cycloheximide (100 μg/ml for 2 h), were rinsed twice with cold PBS containing 1 mM $CaCl_2$, fixed for 5 min on ice with cold PBS–1 mM $CaCl_2$–3.6% formaldehyde, and permeabilized with PBS–0.5% Triton X-100 for 15 min. The *EMS1* protein was detected with preabsorbed and affinity-purified antibodies (ra14 and ra23) conjugated with dichlorotriazinylaminofluorescein (DTAF; Research Organics, Cleveland, Ohio). Talin was detected by using monoclonal antibody 8D4 (Sigma, St. Louis, Mo.) and anti-mouse immunoglobulin-biotin conjugate (Biogenex Laboratories, San Ramon, Calif.) and phycoerythrin-streptavidin (Calbiochem, San Diego, Calif.). The images were taken and analyzed on a Bio-Rad MRC600 confocal microscope.

Nucleotide sequence accession number. The nucleotide sequence of the *EMS1* cDNA has been assigned GenBank accession number M98343.

RESULTS

Cloning of a full-length cDNA encoding the *EMS1* protein. We previously described the construction of a cDNA library from a squamous cell carcinoma cell line with an 11q13 amplification (UMSCC2). Using differential cDNA cloning procedures with cDNA probes generated from mRNA from cell lines with (UMSCC2) or without an 11q13 amplification (UMSCC1), we identified a new gene that was referred to as the *EMS1* gene (42). To obtain full-length cDNA clones, we screened a human T47D cDNA library by using the previously identified 3′ *EMS1* cDNA clone pU21C8B (42). To identify the longest *EMS1* cDNA clone, a 5′ extension cloning procedure was used (Fig. 1a and Materials and Methods). Several overlapping cDNA clones were isolated (Fig. 1a) and sequenced. The complete nucleotide sequence of the *EMS1* cDNA comprises 3,248 nucleotides (41) and contains an open reading frame of 550 codons (Fig. 1b). Several putative functional domains were identified within the predicted *EMS1* protein (Fig. 1c): an internal repeat of 37 amino acid residues (domain A), a proline-serine-threonine-rich domain (domain C), and an SH3 motif (domain D). The SH3 motif is of interest because this motif is commonly

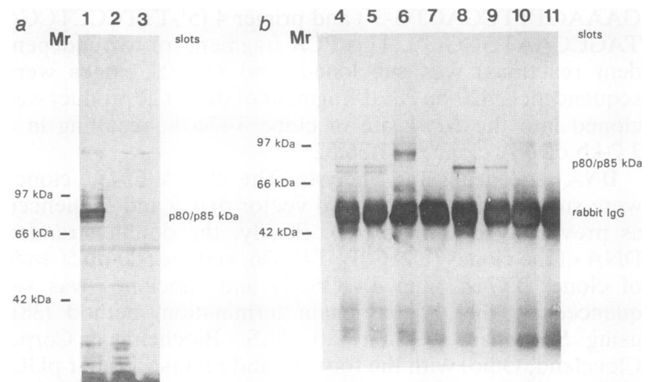

FIG. 2. Detection of human *EMS1* protein in cell lysates. Total lysates of cells with (UMSCC2; lane 1) and without (Jurkat and CEM; lanes 2 and 3, respectively) *EMS1* mRNA expression and immunoprecipitates obtained with either ra14 (lanes 5 and 9) or ra23 (lanes 4 and 8) antiserum from the total lysate of UMSCC2 (lanes 4 to 7) or UMSCC8 (lanes 8 to 11) cells were subjected to PAGE, transferred to PVDF membranes, and incubated with total ra23 antiserum. Reactive proteins were visualized with an anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate. Immunostaining of the same immunoprecipitates with ra14 total antiserum gave identical results (data not shown).

found in proteins that are associated with cytoskeletal proteins such as Src, fodrin, and α-spectrin (29, 30), suggesting that *EMS1* may also interact with cytoskeletal components.

Comparison of the deduced amino acid sequence of the *EMS1* cDNA with sequences available in the GenBank, SWISS-PROT, and NBRF data bases revealed a high homology with two other proteins, a protein encoded by the human *HS1* cDNA (14) and the chicken p80/p85 protein (48). The *HS1* protein contains 450 amino acid residues. To obtain the best homology (overall homology to *EMS1* of 75%), large gaps had to be introduced in the alignment; the highest homology was observed in the tandem repeat and in the SH3 domain (Fig. 1b). This finding suggested that the protein encoded by the *HS1* cDNA is related to but different from the *EMS1* protein. The striking high homology with the chicken p80/p85 protein (an overall identity of 85% with only two gaps in the alignment) and the similar structural features (Fig. 1c) strongly suggest that the *EMS1* protein is the human homolog of chicken p80/p85, which is a substrate for the tyrosine kinase *src* oncogene (12, 48).

Immunodetection of the *EMS1* protein. To characterize the human *EMS1* protein, two rabbit antisera were generated against different domains of the *EMS1* protein (ra14 and ra23 in Fig. 1c). Immunoblotting on cell lysates performed with these antisera showed two prominent characteristic proteins of 80 and 85 kDa (hereafter referred to as the p80/p85 *EMS1* protein) in cell lines that expressed the *EMS1* mRNA (Fig. 2a, lane 1), whereas p80/p85 was not observed in cell lines without detectable *EMS1* mRNA (lanes 2 and 3). To test the specificity of the reaction of these antisera, immunostainings with total antisera and with preabsorbed and affinity-purified antibodies were compared. On preparations of inclusion bodies with the TrpE-*EMS1* ra23 fusion protein, a marked reduction of the reactivity with proteins other than the fusion protein was detected when preabsorbed and affinity-purified ra23 antibodies were used (compare lanes 2 and 4 in Fig. 3a); no reactivity with the TrpE protein (lanes 1 and 3) or the noncorresponding TrpE-*EMS1* ra14 fusion protein was observed. On total lysates of UMSCC8 or UMSCC2 cells, the

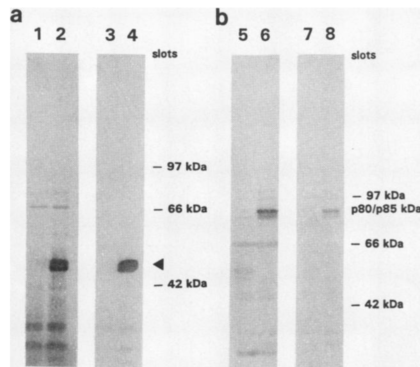

FIG. 3. Characterization of antibodies against the human *EMS1* protein. Immunoblotting of preparations of inclusion bodies with TrpE protein (lanes 1 and 3) or with TrpE–*EMS1* ra23 fusion protein (lanes 2 and 4) and of total lysates of UMSCC8 (lanes 5 and 7) or UMSCC2 (lanes 6 and 8) cells. Protein preparations were analyzed as described for Fig. 2, using total ra23 antiserum (lanes 1, 2, 5, and 6) or preabsorbed and affinity-purified ra23 antibodies (lanes 3, 4, 7, and 8). Incubation with ra14 antiserum gave the same results (data not shown).

reactive proteins other than p80/p85 detected with the total antisera (Fig. 3b, lanes 5 and 6) were considered to be nonspecific, since the immunostaining of these proteins significantly decreased relative to that of p80/p85 when preabsorbed and affinity-purified antibodies were used (lanes 7 and 8). Additional evidence that p80/p85 is the specific antigen for both antisera was obtained from Western blot analysis of immunoprecipitates with subsequent immunoblotting with either ra14 or ra23 antiserum. In immunoprecipitates obtained with use of the ra14 antiserum, a p80/p85 protein could be detected with the ra23 antiserum (Fig. 2b) and vice versa (not shown). Furthermore, the relative amount of the p80/p85 *EMS1* protein detected by immunoblotting of epithelial carcinoma cell lines with (UMSCC2, UMSCC22B, and MDA-MB-134-IV) and without (UMSCC1, UMSCC8, T47D, and HBL100) amplification (e.g., Fig. 3b) corresponded to the level of gene amplification and mRNA overexpression of the *EMS1* gene in these cells (40, 42). These data suggest that both the p80 and p85 proteins contain the sequences recognized by the two specific antisera directed against distinct *EMS1* sequences and that both proteins most likely are encoded by the same gene. Similar observations were described for antibodies directed against the chicken p80/p85 protein (48).

Subcellular localization of the *EMS1* protein. In nontransformed chicken embryo fibroblasts, the p80/p85 protein was described to be localized at the cell periphery; transformation of the cells with Rous sarcoma virus resulted in an accumulation of p80/p85 in podosomes (48), clusters of small rosette adhesion sites (6, 7, 44) containing characteristic adherens-type junction components such as vinculin, talin, α-actinin, and F-actin (6, 7, 28, 44). To study the subcellular localization of the human p80/p85 *EMS1* protein, we performed immunocytochemistry with the two preabsorbed and affinity-purified antibodies on carcinoma cell lines with and without 11q13 amplification. The two affinity-purified antibodies revealed similar patterns of immunofluorescent staining. The reactivity of both antibodies with the *EMS1* protein was specific, since it could be blocked with the corresponding fusion protein that was used to generate the antisera, while the noncorresponding *EMS1* fusion protein had no effect on the antibody reactivity (not shown).

In a squamous cell carcinoma cell line with an 11q13 amplification (UMSCC22B), an intense cytoplasmic staining was observed (Fig. 4a). However, the *EMS1* protein was also found in dot-like outgrowths at the cell membrane, similar to podosome-like structures (arrow in Fig. 4a). Double-staining experiments showed a colocalization of the *EMS1* protein and talin in these podosome-like structures (Fig. 4a and b, respectively). Vinculin, an other adherens-type junction-associated protein (6, 27), also colocalized in the podosome-like structures (data not shown). However, both the *EMS1* protein (open arrowheads in Fig. 4c and d) and talin were not observed in intercellular contact sites, whereas vinculin was detected at these sites (not shown). This finding indicates that in epithelial cells, the *EMS1* protein is localized in talin-containing substratum-adherens-type junctions. After treatment of the UMSCC22B cells with cycloheximide to block de novo protein biosynthesis, the intense cytoplasmic staining decreased dramatically (Fig. 4c). However, some punctate staining could still be detected in the cytoplasm, suggesting that a fraction of the protein might be associated with cytoskeletal components, but the main proportion of the *EMS1* protein was now localized in podosome-like structures (arrows in Fig. 4c). A similar staining pattern was observed in two other cell lines with an 11q13 amplification (UMSCC2 and MDA-MB-134-IV).

In an epithelial cell line without an 11q13 amplification, UMSCC8 (42), a diffuse cytoplasmic localization of the *EMS1* product was observed (Fig. 5a), with some sporadic staining in the protruding leading lamellae (arrowhead) that are considered to be the primordial contacts in the focal adhesion sites (32). Double staining of UMSCC8 cells with antibodies detecting the *EMS1* protein and talin again showed a colocalization of the two proteins (Fig. 5a and b, respectively). After the cycloheximide treatment, the pattern of subcellular staining of the *EMS1* protein was similar to that in nontreated cells; the protein was still observed mainly in the cytoplasm (Fig. 4d), with only some sporadic staining in the protruding leading lamellae (closed arrowheads). A similar staining pattern was detected in three other epithelial cell lines without an 11q13 amplification (T47D, UMSCC1, and HBL100). This staining pattern differed markedly from the distribution observed in epithelial cells with an 11q13 amplification (compare Fig. 4c and d).

DISCUSSION

We have previously identified two genes (*EMS1* and *PRAD1*/cyclin D1) in the chromosome 11q13 region that are frequently coamplified and overexpressed in human breast cancer and in squamous cell carcinomas of the head and neck (42). In this report, we demonstrate that the protein encoded by the *EMS1* gene shows the highest amino acid sequence homology (identity of 85%) to the chicken p80/p85 protein that was recently identified as a substrate for the *src* oncogene (48). To characterize the human protein, we have used two antisera directed against different domains in the *EMS1* protein and demonstrated that both of these antisera recognize an 80- and an 85-kDa protein (Fig. 2 and 3), similar to what has been described for the chicken p80/p85 protein. As illustrated in Fig. 3b, the relative amount of cellular p80/p85 protein was higher in cells with an 11q13 amplification than in cells without 11q13 amplification. The levels of p80/p85 *EMS1* protein correlated with the levels of 11q13 amplification and mRNA overexpression of the *EMS1* gene in these cell lines (40, 42).

Immunocytochemistry revealed a substantial difference in

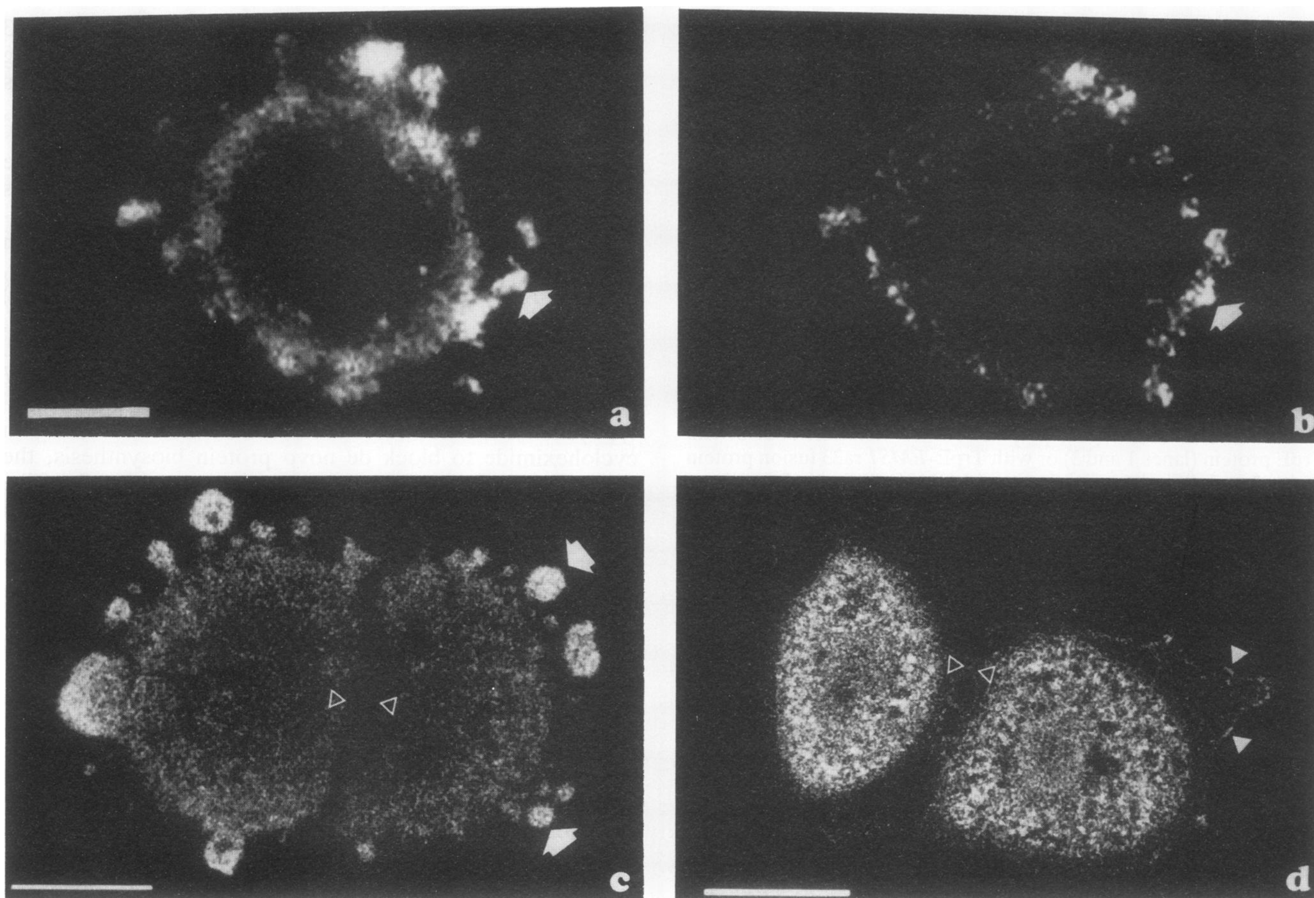

FIG. 4. Immunolocalization of the *EMS1* protein in subcellular structures of different squamous cell carcinoma cell lines. Cells with an 11q13 amplification (UMSCC22B; a to c) and cells without amplification (UMSCC8; d) were grown in the absence (a and b) or presence (c and d) of cycloheximide and stained simultaneously with preabsorbed and affinity-purified DTAF-conjugated ra23 antibodies directed against *EMS1* (a, c, and d) and with a murine anti-human talin monoclonal antibody (b) as described in Materials and Methods. In the double-staining experiment, both images of the same location for staining of *EMS1* (a) and for talin (b) are presented. Symbols: arrows, podosome-like structures; closed arrowheads, staining in the protruding leading lamellae; open arrowheads, intercellular contacts. The images were taken and analyzed on a Bio-Rad MRC600 confocal microscope. Bars represent 50 (a), 25 (d), and 10 (c) μm.

subcellular localization of *EMS1* between human carcinoma cells with and without 11q13 amplification. In cells without the amplification, most of the *EMS1* protein is localized in the cytoplasm, whereas only a sporadic fraction of the protein is localized in the protruding leading lamellae at the periphery of the cell. On the other hand, in cell lines with an 11q13 amplification, *EMS1* accumulated not only at the periphery (compare Fig. 4c and d) but also in distinct structures at the periphery, in podosome-like structures. The localization of *EMS1* at the periphery in carcinoma cell lines either with or without 11q13 amplification was similar to the pattern of talin, the substratum-adherens-type junction protein (5, 6, 27, 28). The distribution of the *EMS1* protein in human epithelial cell lines with or without 11q13 amplification is comparable to the redistribution of the chicken p80/p85 protein in normal or $p60^{src}$-transformed fibroblasts, respectively. In nontransformed chicken embryo fibroblasts, p80/p85 showed a primarily cytoplasmic localization, with only some sporadic staining at the periphery, whereas in $p60^{src}$-transformed fibroblasts, p80/p85 accumulates in podosome-like structures (48). Our findings, therefore, suggest that the *EMS1* protein may play some important role in the organization of the cytoskeleton and/or of the cell adhesion

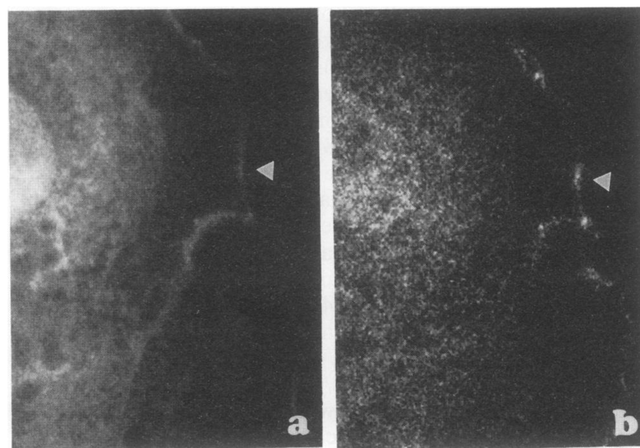

FIG. 5. Colocalization of the *EMS1* protein with talin in a cell line without the *EMS1* gene amplification. Attached cells without amplification (UMSCC8) were fixed, permeabilized, and stained with affinity-purified DTAF-conjugated ra23 antibodies (a) and with an anti-human talin monoclonal antibody (b) as described for Fig. 4. Both images of the same location are presented. Arrowheads indicate staining in the protruding leading lamellae.

structures of human epithelial and carcinoma cells. This observation is in good agreement with the findings described for the chicken p80/p85 protein, which is considered the chicken homolog of the human *EMS1* protein (this report). Recently, additional evidence for the possible role of the chicken p80/p85 protein in the organization of the cytoskeleton and/or of the cell adhesion structures was described by Wu and Parsons (49), who showed that the chicken p80/p85 protein specifically associates with F-actin.

We observed a correlation between 11q13 amplification with concomitant overexpression of the *EMS1* protein and the presence of podosome-like structures in carcinoma cell lines. Podosomes have been detected in many transformed cells as well as in normal cells such as spreading cells of monocytic origin and osteoclasts (22), but they are most prominent in cells transformed by viruses harboring oncogenes encoding tyrosine kinases (6, 7, 44) such as $p60^{v\text{-}src}$, $p120^{v\text{-}abl}$, $p130^{v\text{-}fps}$, and $p80/90^{v\text{-}yes}$. These podosome-like structures also contain substrates for tyrosine kinases such as p110/120, vinculin, and talin (6, 7, 12, 44) as well as various kinases ($p125^{fak}$, $p60^{v\text{-}src}$, $p80/90^{v\text{-}yes}$, and $p120^{v\text{-}abl}$) (6, 10, 34, 35, 39, 46, 52). Chicken p80/p85 is a substrate for the $p60^{v\text{-}src}$ oncogene (12, 48). It is assumed that the observed accumulation of p80/p85 in podosomes is the result of the morphological transformation of chicken embryo fibroblasts by $p60^{v\text{-}src}$ rather than a cause of the transformation of these cells. Analogously, the redistribution of *EMS1* in human carcinoma cells with an 11q13 amplification may not be the cause but merely an effect of podosome formation, and as such interfere with the organization and the functioning of cytoskeleton and cell adhesion structures, and consequently could well affect cell-matrix contacts. Since all cell lines used in this study are derived from carcinomas, the redistribution of the *EMS1* protein associated with gene amplification might be involved in differences in cell adhesive and/or invasive properties between the different cell lines. Since amplification of the 11q13 region, harboring the *EMS1* gene, has been associated with an increased invasive and metastatic behavior in human breast carcinomas (1, 2, 13, 41, 43), the *EMS1* gene might mediate these properties. It remains to be proven whether such direct link between 11q13 amplification, overexpression of *EMS1*, relocalization of *EMS1* in podosome-like structures, and invasive behavior of tumor cells exists.

The accumulation of the chicken homolog, the p80/p85 protein, in podosomes in Rous sarcoma virus-transformed chicken fibroblasts was described to be accompanied with tyrosine phosphorylation rather than an increased expression of the protein (12, 48). This finding might suggest that overexpression of the *EMS1* protein by itself is not sufficient for the redistribution in human carcinoma cells with 11q13 amplification. The presence of numerous potential phosphorylation sites in the human *EMS1* protein (Fig. 1c) provides multiple possibilities for such posttranslational modifications. A kinase that might be one of the candidate modifiers of the *EMS1* protein is cdk4, a serine/threonine kinase that preferentially associates with *PRAD1*/cyclin D1 (23, 51). cdk4 might be involved since its regulator, *PRAD1*/cyclin D1, is located on the same 11q13 amplicon at a distance of 800 kb from the *EMS1* gene (4) and is overexpressed and coamplified with *EMS1* in carcinomas with an 11q13 amplification (42). Whether phosphorylation triggered by cdk4 or other kinases is indeed involved in the altered localization of the *EMS1* protein in epithelial tumor cells and which kinases are responsible for this relocalization is a subject for further analysis.

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