Identification of the Motility-related Protein (MRP-1), Recognized by Monoclonal Antibody M31-15, Which Inhibits Cell Motility

By Masayuki Miyake,* Masaru Koyama,† Masaharu Seno,‡ and Shuichi Ikeyama†

From the *Department of Thoracic Surgery, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka 530; and †Biotechnology Research Laboratories, Research and Development Division, Takeda Chemical Industries, Ltd., Osaka 530, Japan

Summary

A murine monoclonal antibody (M31-15) was identified using the penetration-inhibiting assay of a human lung adenocarcinoma cell line (MAC10) and remarkably inhibited the phagokinetic track motility of various cancer cell lines. The antigen, motility-related protein (MRP-1), recognized by M31-15, was 25- and 28-kD proteins, and M31-15 was used to isolate a cDNA clone from a human breast carcinoma cDNA library. Sequence analysis revealed that MRP-1 had strong similarity with a B cell surface antigen (CD37), a melanoma-associated antigen (ME491), the target of an antiproliferative antibody (TAPA-1), a human tumor-associated antigen (CO-029), and the Sm23 antigen of the trematode parasite Schistosoma mansoni.

Cell motility is a highly complex process dependent on pericellular adhesion molecules such as fibronectin (FN) and laminin, their integrin receptors, cytoskeletal components, and a junctional unit connecting cytoskeletal components and membrane receptors (1, 2). Involvement of the cell-binding and Hep-2 domains of FN and integrin receptor in determining cell motility for certain types of cells has become increasingly clear (3). In addition, a large variety of growth factors and autocrine factors have been shown to stimulate cell motility (4). There has been little attention paid to carbohydrates that modulate cell motility despite the fact that cell surface proteins and lipids are heavily glycosylated. Recently, we (5) showed that two independent mAbs directed to a common carbohydrate structure, Fuca 1-2Galβ1-R, dramatically inhibited the motility of various cancer cell lines and metastasis of BL6 (6), highly metastatic variants of mouse melanoma B16. To identify cell surface molecules involved in the control of cell motility, we described a new approach based on: (a) selection of a few lines showing high motility out of a large number of human tumor cell lines; (b) immunization of mice with the high-motility cell lines and selection of mAbs showing inhibition of cell motility in polycarbonate membrane penetration assay; and (c) characterization of the epitope defined by motility-inhibiting mAbs. This procedure has led us to an interesting mAb, M31-15, which recognized 25- and 28-kD cell surface proteins called motility-related protein (MRP-1). A cDNA clone for MRP-1 was isolated from a human breast carcinoma cDNA library with this antibody, and the amino acid sequence of MRP-1 was predicted from the cDNA sequence.

Materials and Methods

Determination of Cell Motility. Cell motility was determined by two assay systems, cell penetration (5) and phagokinetic track (7). In the former system, cell migration was determined through a polycarbonate membrane assembly based on a Boyden chamber (1, 8). The assembly (Transwell) was purchased from Costar (Cambridge, MA) with defined pore size (3-, 5-, and 8-μm diameter). Briefly, 100-μl hybridoma supernatant containing a known concentration of mAb was mixed with 600 μl of culture medium in the lower Transwell compartment, and 10^5 cells were placed in the upper chamber and cultured for 16 h. Cells penetrating into the lower chamber, found mostly (95%) at the bottom of the lower compartment, were then counted. In the phagokinetic track assay, cell motility was determined on the basis of phagokinetic tracts on gold particle-coated plate (7). Uniform carpets of gold particles were prepared on glass coverslips coated with BSA, and the coverslips were rinsed extensively to remove nonadhering or loose gold particles before cell plating. 1,000-2,000 freshly trypsinized cells were plated in a 3.5-cm plastic dish (Falcon Labware, Oxford, CA) and left in the incubator for 16 h. Phagokinetic tracks were visualized in dark-field microscopy at low power by using side illumination. Gold particles were detected as dark dots. Pictures were taken, areas cleared by a single cultured cell were traced on semitransparent paper of uniform thickness, and average areas were calculated (9). Since this method only measures minimum motility, it was used.

Abbreviations used in this paper: FN, fibronectin; MRP-1, motility related protein.
only for confirmation of penetration assay results. On the other hand, motility of many types of cells cannot be measured by penetration assay, since these cells hardly migrate through polycarbonate membrane. However, motility of most these cells can be measured easily by phagokinetic track assay.

Selection of mAbs Displaying Inhibition of Cell Motility. Initially, cell motility of >120 human tumor cell lines was examined by the penetration assay described above. Only a few lines were found to be highly motile: U937 (histiocytic lymphoma) (10), HEL (erythroleukemia) (11), and MAC10 (lung adenocarcinoma) (5). U937 and HEL were able to penetrate Transwell membranes with pore diameters of 3 μm, and MAC10 can do 5 μm, respectively. All other tumor cell lines tested were less motile, and normal cells were immotile. Initially, mice were immunized with MAC10 cells and a few mAbs inhibiting MAC10 motility were obtained, but all of these antibodies were detecting specific epitopes that only MAC10 cells had. We could not find any other cancer cell lines on which such epitopes were expressed. As we wanted to find new motility-inhibiting factors that are common to many kinds of solid cancer cell lines, we decided to immunize mice with cancer cells different from MAC10 to be used for the Transwell penetrating assay. Subsequently, mice were immunized with MAC8 (lung adenocarcinoma), which originated from brain metastasis lesion, and their splenocytes were fused with HAT-sensitive mouse myeloma cell line SP2 as described previously (12). Antibody-secreting hybridomas were selected on the basis of their ability to inhibit cell motility (see above) in the motile cell lines. Inhibition of cell motility was expressed as the reduction of the number of cells able to penetrate the polycarbonate membrane.

Determination of mAb Specificity. Glycolipids were extracted from MAC10 cells with isopropanol/hexane/water (55:20:25; vol/vol/vol) (13) and partitioned according to Folch-Pi et al. (14). The glycolipids in the upper layer fraction of the Folch-Pi’s partition were subjected to DEAE Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography. After the neutral glycolipids were eluted with chloroform/methanol/water (30:60:8; vol/vol/vol), gangliosides were eluted with chloroform/methanol/0.8 N sodium acetate (30:60:8; vol/vol/vol) (15). Such two-mixture fractions were used in the ELISA and TLC immunostaining method without further purification (16, 17). The antibodies selected were tested against extracted glycolipids coated on plastic plates, as well as protein (or glycoprotein) in the cellular extracts with 1% triton X-100 coated on plastic surface and tested for antibody binding. Glycoprotein or protein antigens were also characterized after cells were extracted with Laemmli’s sample buffer (18) followed by slab gel electrophoresis, Western blotting, and immunostaining (19). Alternatively, cells were washed with 0.1 M Tris/10 mM EDTA/0.2 mM (p-amidinophenyl) methansulfonyl fluoride (pH 8.1) and solubilized with 1% CHAPS in the above buffer for 2 h on ice. Insoluble materials were removed by centrifugation, and an aliquot of the soluble fraction was subjected to slab gel electrophoresis followed by Western blotting and immunostaining (18, 19).

Antibody Binding to Various Cell Lines. A variety of motile and immotile human cell lines, as well as high-metastatic (BL6) and low-metastatic (F1) variants of mouse melanoma cell line B16 (6, 20), were tested by immunofluorescence with FITC-conjugated goat anti-mouse IgG (Tago Inc., Burlingame, CA) and by cytofluorometry (21).

Immunohistochemical Techniques. The avidin-biotin complex techni
cques for immunohistochemical study of various kinds of cancers was performed as follows (22): tumor sections of 4-μm thickness were made free of paraffin by soaking in xylene and dehydrating in graded ethanol. The endogenous peroxidase activity was blocked by treating the sections with 0.3% hydrogen peroxide for 20 min. After washing for 5 min in 50 mM Tris buffer, pH 7.4, the sections were incubated for 2 h at room temperature with normal horse serum and then exposed overnight to the culture supernatant of the produced hybridoma. After washing in 50 mM tris buffer for 20 min, the sections were treated for 2 h with the solution of biotinylated horse anti-mouse IgG, washed again for 20 min, and treated for 1 h with PBS that contained the avidin-biotin complex. After washing in 50 mM Tris buffer for 30 min, the sections were incubated with the substrate solution, which contained 3,3'-diaminobenzidine for 1 min and washed with water and weakly counterstained with hematoxylin. Sections that had been incubated with SP2 culture supernatant served as a negative control. Biotinylated horse anti-mouse IgG, avidin, and biotin were purchased from Vectastain (Burlingame, CA).

Cloning and DNA Sequencing. A λgt11 expression cDNA library prepared with mRNA from human breast carcinoma cell line ZR-75-1 (Clontech, Palo Alto, CA) was screened with M31-15 as described (23). DNA of the resulting phage clone, MRP-1, was purified and the cDNA portion (1.1-kb fragment) was excised with EcoRI and recloned into the EcoRI site of plasmid pUC18 at (24) to construct plasmid pBT1352. Cloned cDNA was digested with appropriate restriction endonucleases to generate small fragments. The fragments were subcloned into plasmid pUC18 and pUC119 vectors. Single-stranded DNA was prepared by transfecting helper phage KO7, and the nucleotide sequences were determined by the dideoxynucleotide chain termination method (25) using appropriate synthetic oligonucleotide primers. Both strands were then sequenced and all restriction sites used for subcloning were crossed. The sequence similarity of MRP-1, TAPA-1, CO-029, ME491, CD37, and Sm23 cDNA was identified by a computer search of GenBank Genetic Sequence Database rel 67 using MSAP (Mitsui Knowledge Ind., Tokyo, Japan).

Results

Selection of mAb M31-15, Inhibiting MAC10 Motility. Hybridomas were produced after immunization of BALB/c mice with MAC8 cells. About 3,000 hybridoma supernatants were screened in a penetration assay that measured the ability of MAC10 cells to penetrate the Transwell membrane pores (5). mAb M31-15 (isotype IgGl) was found to show the strongest effect on inhibition of MAC10 motility. The effect of mAb concentration was dose dependent and led to constant inhibition at 1 μg/ml (Fig. 1). Control mouse IgG showed no inhibitory effect on cell motility regardless of concentration.

Inhibition of Phagokinetic Motility of Various Cancer Cells by mAb M31-15. Phagokinetic motility of various kinds of cancer cells was tested using the gold particle coating method described previously (7). Areas of the particle-clear zone for cancer cells were measured after a 16-h incubation, and the average area was calculated. The area of tracks swept out by the moving cells was reduced by 50–77%, as compared with the controls (Table 1). The controls contained mouse IgG (1 μg/ml) instead of mAb M31-15. A typical example of inhibition of MAC10 phagokinetic motility in the presence of M31-15 is shown in Fig. 2.

Expression of MRP-1 on Various Kinds of Cells by Cytofluorometry. A variety of cancer and fetal cell lines were analyzed...
Figure 1. Dose-dependent effect of mAb M31-15 on cell penetration. Cell penetration was determined with MAC-10 cells in the lower chamber of Transwell. The constant inhibitory effect was produced by M31-15 concentrations of 1 μg/ml.

The estimated antibody concentration was 0.01 to 0.32 μg/ml.

Table 1. Inhibitory Effect on Phagokinetic Motility of Various Cancer Cell Lines by M31-15

| Cell line | Control | M31-15 |
|-----------|---------|--------|
|           | μm² ± SD|        |
| MAC10     | 9,820 ± 3,010 | 2,250 ± 760 |
| QG-90     | 4,310 ± 1,730 | 1,650 ± 690 |
| PC-9      | 5,320 ± 1,950 | 1,610 ± 590 |
| SK-LU-1   | 3,510 ± 1,050 | 1,490 ± 420 |
| KATOIII   | 4,250 ± 1,640 | 2,130 ± 820 |

Estimated by average area of tracks on gold particle-coated surface, as described in Materials and Methods. Data are calculated from 100 unselected cells. Mouse IgG was used as control at the same concentration (1 μg/ml).

Figure 2. Effect of mAb M31-15 on motility of MAC-10 cells as determined by pharmacokinesis from the gold colloid method. (a) MAC-10 cell motility in medium containing M31-15 (1 μg/ml). (b) MAC-10 cell motility in medium containing mouse IgG (1 μg/ml).
Table 2. Surface Expression of Various Cell Lines

| Cell line   | Histology                          | Surface expression |
|-------------|------------------------------------|--------------------|
| MAC10       | Lung adenocarcinoma                | + +                |
| PC-14       | Lung adenocarcinoma                | + +                |
| PC-9        | Lung adenocarcinoma                | + +                |
| A-549       | Lung alveolar cell ca.             | + +                |
| KATOIII     | Gastric ca.                        | + +                |
| AZ521       | Gastric ca.                        | + +                |
| PLC/PRF/5   | Hepatocellular ca.                 | -                  |
| HEP-G2      | Hepatocellular ca.                 | -                  |
| MIA PaCa-2  | Pancreatic ca.                     | + +                |
| BxPC-3      | Pancreatic ca.                     | + +                |
| ZR-75-30    | Breast ca.                         | + + +              |
| MCF-7       | Breast ca.                         | + +                |
| COLO201     | Colon ca.                          | + +                |
| Hela        | Uterine Ca.                        | + +                |
| KB          | Nasopharyngeal ca.                 | + +                |
| A-431       | Epidermal ca.                      | +                  |
| HEL         | Erythroleukemia                    | +                  |
| IM-9        | Lymphoblastoma                     | -                  |
| HL-60       | Promyelocytic leukemia             | -                  |
| J111        | Monocytic leukemia                 | + +                |
| U266        | Myeloma                            | -                  |
| Daudi       | Burkitt's lymphoma                 | -                  |
| Raji        | Burkitt's lymphoma                 | -                  |
| G361        | Melanoma                           | +                  |
| T98G        | Glioma                             | + +                |
| Flow 7000   | Fibroblast                          | + +                |
| WI-38       | Fetus lung                          | -                  |
| IMR-90      | Fetus lung                          | -                  |
| WISH        | Human amnion                        | + +                |
| MEG-01      | Megakaryocyte                       | -                  |
| CEM         | T cell line                         | -                  |
| F1          | Mouse melanoma                      | -                  |
| BL6         | Mouse melanoma                      | -                  |

Determined by flowcytometry. Mean channel fluorescence intensity compared with negative control staining according to the following scale: -, 0-5%; ±, 5-10%; +, 10-50%; + +, 50-90%; + + +, >90%.

resolved by both ELISA and TLC immunostaining (data not shown). However, on Western blotting of the soluble fraction of MAC10, two bands (25 and 28 kD) were clearly demonstrated (Fig. 3). These two bands were detected from various cancer cells extracts (Fig. 3). Digestion of MAC10 cell extracts with periodate had no effect on the migration and intensity of the two bands (data not shown), so these two bands were thought to be not glycosylated.

Isolation of the MRK-1 cDNA. Using M31-15, the cDNA clone AMRP-1 was isolated from a Agt11 expression cDNA library prepared with mRNA from human breast carcinoma cell line ZR-75-1. The length of the insert carried in the phage vector was ~1.1 kb when checked by PCR with universal primers specific to the phage DNA designed to amplify the insert cDNAs. Partial nucleotide sequencing of this amplified fragment showed the direction of the cDNA insert. For further analyses, the cDNA prepared from phage DNA was subcloned into the plasmid pUC118 (24).

Sequence Analysis of MRK-1 cDNA. The nucleotide sequence of a 1,120-bp MRK-1 cDNA in AMRP-1 was determined as shown in Fig. 4. A search of the genetic sequence database (GenBank rel. 67) revealed a striking partial sequence similarity of MRK-1 to the members of a transmembrane protein family consisting of TAPA-1 (26), CO-029 (27), ME491 (28), CD37 (29), and Sm23 (30) (Fig. 5). The similarly of MKP-1 to them was 43.5%, 35.1%, 26.4%, 22.3%, and 25.5%, respectively. The open reading frame of the MRK-1 cDNA was speculated by considering that of the β-galactosidase gene of Agt11 and comparing those of the members of the transmembrane protein family. The first ATG (at 112 in Fig. 4) of the predicted open reading frame is flanked by sequences (TCACCATGC) that fulfill the criteria for initiation codons (CCA/GCCATGG) proposed by Kozak in 1984 (31). This open reading frame of MRK-1 encodes a potential polypeptide of 228 amino acid residues with a predicted molecular weight of 25,419, which is consistent with the molecular mass of the bands at 25 and 28 kD detected in Western blotting described above.

Discussion

Metastasis is among the most important problems in the therapy of the patients with various kinds of cancers. Almost all patients with disseminated cancer are incurable by cur-
Figure 4. Nucleotide and deduced amino acid sequence of MRP-1 cDNA. The nucleotide sequence of the L1-kb MRP-1 cDNA was determined as described in Materials and Methods. The amino acid sequence encoded by the open reading frame is shown using the single-letter amino acid code. Possible asparagine-linked glycosylation site is underlined.

Figure 5. Amino acid sequence similarities. MRP-1 was aligned with the CO-029, ME491, CD37, TAPA-1, and Sm23 proteins. Identities between any four of the proteins are boxed. Gaps were created by MSAP program to permit best alignment. The predicted transmembrane domains proposed by Szala et al. (27) are overlined.
recently known methods, and the metastatic dissemination of
tumor cells to secondary distinct sites requires highly motile
behavior. Despite various kinds of specific antitumor defense
mechanisms, tumor cells surviving in the blood and lymph
vessels must be resistant to the shear stresses arising in the
vascular bed, the frictional forces arising between their periph-
ery and vessel walls, as well as have the ability to traverse
capillaries that generally are rigid and smaller in diameter than
tumor cells (2, 32). However, the biochemical mechanisms
regulating such invasive tumor cell motility are poorly un-
derstood. Motility is an essential cellular function closely
related to the process of tumor metastasis and at least plays
a very important role in tumor invasion and metastasis. Inhi-
bition of tumor cell motility may have the possibility of leading
to conquering tumor cell metastasis.

Birchmeier et al. (33) studied the correlation between cell
adhesion and metastatic potential of tumor cells by use of
mAbs that inhibit these phenotypes. They found that vari-
ous mAbs affected phenotypic characteristics of melanoma
cells such as adhesion, growth, migration, and morphology,
but the molecular basis of the effect of these mAbs was not
clearly shown. In the present study, this approach was used
to establish motility-inhibiting mAbs after immunization of
mice with human cancer cell lines characterized by high mo-
tility. Screening and selection of mAbs were performed based
on the inhibition of tumor cell motility as measured by a
modified Boyden Transwell chamber. Through this proce-
dure, we obtained various mAbs that inhibited penetrating
activity; M31-15 showed the strongest inhibition. M31-15
reacted with most human cancer cell lines, especially solid
tumor cell lines (Table 2), and many of them expressed two
25- and 28-kD proteins detected by Western blotting (Fig.
3). These two species were not affected by the peroxidase
digestion, suggesting that MRP-1 was not glycoslated (data
not shown). The predicted molecular mass of MRP-1 is 25.4
kD, which coincided with the protein detected at 25 kD.
The protein detected at 28 kD suggests the existence of the
other member of the family, which is immunoreactive with
M31-15. This 28-kD species may be the NH2-terminal ex-
tended form of MRP-1 translated from the other protein (CTG
at 43) upstream of the first ATG at 112, as shown in the case
of basic fibroblast growth factor gene (34).

The mechanism of motility inhibition by M31-15 is not
clear. MRP-1 might be a receptor for a yet-to-be discovered
member of the integrin family. The sequence of MRP-1 does
not show any similarity to known integrin family receptors,
but shows striking partial similarity to CD37, ME491,
TAPA-1, CO-029, and Sm23 (26) (Fig. 5). Very recently these
molecules were found to form a new family of transmem-
brane type cell surface proteins (26). CD37 is highly expressed
in human B cells and at lower levels in other hematopoietic
cell types (35). Anti-CD37 antibodies have been shown to
inhibit the mitogenic effect of anti-CD20 plus B cell growth
factor on B cells as well as stimulate the mitogenic effect
of anti-Ig antibodies on these cells (36). ME491 is expressed
during the early stages of melanoma but is downregulated
during later stages of melanoma, which are more aggressive
and adaptable to tissue culture (37). TAPA-1 is the cell sur-
face protein expressed on most but not all human cell lines,
and this antibody has a marked antiproliferative effect on many
lymphoid cell lines (26). CO-029 antigen is found to be ex-
pressed on gastric, colon, rectal, and pancreatic carcinoma,
except on most normal tissues (38). mAbs against CO-029
have been shown to mediate antibody-dependent cell-mediated
cytotoxicity in vitro, but had no effect on the growth of human
colorectal carcinoma and melanoma lines (39). Sm23 is found
as the integral membrane protein of Schistosoma mansoni. The
function of Sm23 has not been clarified (30). The amino acid
sequence of MRP-1 shows the highest similarity to TAPA-1
at 43.5%, but unlike TAPA-1, MRP-1 has little effect on cell
growth of some kinds of tumor cell lines, including MAC10
(data not shown). As shown in Table 1 and Figs. 1 and 2,
MRP-1 is thought to be closely related to motility. MRP-1
is expressed on many but not all cancer cell lines (Table 2),
and M31-15 inhibits motility of some of these cell lines in
the phagokinetic tract assay (Table 1). It is unknown whether
CD37 (29), ME491 (28), TAPA-1 (26), and CO-029 (27) have
effects on cell motility. We are going to study what kind
of structure common to these families has an essential effect
on cell motility with synthetic peptides.

We thank Prof. S. Hakomori (University of Washington), and Drs. A. Kakinuma and K. Igarashi (Bio-
technology Research Laboratories, Takeda Chemical Industries) for their advice and encouragement
throughout this work.

Address correspondence to Masayuki Miyake, Kitano Hospital, Tazuke Kofukai Medical Research Insti-
tute, 13-3, Kamiyamacho, Kitaku, Osaka, Japan.

Received for publication 13 June 1991 and in revised form 12 August 1991.

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