Structure and Function of Band4.1 Superfamily Members

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ABSTRACT

Band4.1 protein is one of peripheral membrane proteins of erythrocyte membranes, which directly binds to glycophorin C, an integral membrane protein, at its N-terminal half. Recently, the amino acid sequence of the N-terminal half of this protein was found to be conserved in the N-terminal end of several distinct proteins, pointing to the existence of the band4.1 superfamily. In addition to band4.1 protein, this band4.1 superfamily includes ezrin, radixin, moesin, talin and protein-tyrosine-phosphatases (PTPH1 and PTPMEG). Furthermore, the tumor suppressor protein for neurofibromatosis 2 named 'merlin' is also reportedly included in this superfamily. For a better understanding of the structure and functions of these superfamily members, I first tried to clarify the more detail picture of this superfamily using polymerase chain reaction (PCR) methods (Chapter I), and next to examine the physiological functions of ezrin/radixin/moesin by the use of antisense oligonucleotides (Chapter II).

In the Chapter I , in order to examine the structural diversity of this superfamily members, using the PCR method with synthesized mixed primers, I have attempted to list up as many members of the band4.1 superfamily as possible expressing in mouse teratocarcinoma F9 cells and the mouse brain tissue. In total, 14 distinct types of cDNA clones were obtained; 8 clones were identical to the corresponding parts of cDNAs for the so far identified members, while 6 clones appeared to encode novel members (NBL1-6 : Novel Band4.1—like proteins). Sequence analyses of these clones revealed that the band4.1 superfamily can be subdivided into 6 gene families such as band4.1 protein, ERM (ezrin/ radixin/ moesin), talin, PTPH1 (PTPH1/ PTPMEG/ NBL1-3), and NBL4 (NBL4/ NBL5) and merlin (merlin/ NBL6) families. The existence of the NBL4 family was first recognised here. I screened F9 cell cDNA library and obtained a full length (2.5-kb) cDNA encoding an NBL4. NBL4 cDNA contains an open

reading frame of 554 amino acids.   Its N-terminal half segment is more homologous to those of the PTPases and band4.1 protein than the other superfamily members.   Its band4.1 homology domain bears a putative myristoylation site and phosphorylation sites for A-kinase and protein-tyrosine kinases, suggesting its possible involvement in the regulation of cellular events just beneath the plasma membrane.   In this study, I describe initial characterization of these new members and discuss the evolution of the band4.1 superfamily.

In the Chapter II, to examine the   functions of ERM family members, antisense oligonucleotides to the N-terminal sequences of ERM family members were constructed and added to cultures of mouse epithelial cells (MTD-1A cells) and thymoma cells (L5178Y), which coexpress all the members.   Immunoblotting revealed that each antisense oligonucleotides selectively suppressed the expression of the corresponding member to the undetectable level.   Immunofluorescence microscopy of these ezrin, radixin or moesin "null" cells showed that all the ERM family members are colocalized at cell-to-cell adherens junctions, microvilli and cleavage furrows where actin filaments were densely associated with plasma membranes.   The ezrin/radixin/moesin antisense oligonucleotides mixture induced the destruction of both cell-cell and cell-substrate adhesion and the disappearance of microvilli. Ezrin or radixin antisense oligonucleotides singly affected the initial step of the formation of both cell-cell and cell-substrate adhesion, but showed no effects on the microvilli structures.   In sharp contrast, moesin antisense oligonucleotide did not singly show any effect on cell-cell and cell-substrate adhesion, whereas it partly affected the microvilli structure.   These data indicate that ezrin and radixin can be functionally substituted for each other, that moesin has some synergetic functional interaction with ezrin and radixin, and that these ERM family members are directly involved in cell-cell and cell-substrate adhesion as well as microvilli formation.

Chapter I

Structural Diversity of Band4.1 Superfamily Members

Introduction

The band 4.1 superfamily includes several distinct proteins which contain the conserved domain homologous to the N-terminal half of band 4.1 protein (see for a review, Tsukita et al., 1993). Band 4.1 protein is one of the major constituents of the undercoat of erythrocyte membranes (Bennett, 1989). This protein forms a complex with spectrin molecules and short actin filaments (Fowler and Taylor, 1980; Ohanian et al., 1984; Ungewickell et al., 1979) and binds to an integral membrane protein called glycophorin C (glycoconnectin) (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985; Bennett, 1989; Leto et al., 1986; Mueleer and Morrison, 1981). The N-terminal half of band 4.1 protein is responsible for this glycophorin C-binding (Leto et al., 1986).

The member which was first reported to belong to this superfamily is ezrin. Ezrin (also called cytovillin or p81) was originally identified as a protein localizing just beneath the plasma membrane of intestinal micro-villi (Bretscher, 1983; Pakkanen et al., 1987), and was revealed to be a good substrate in vivo for some tyrosine kinases (Bretscher, 1989; Gould et al., 1986; Hunter and Cooper, 1981, 1983). Sequence analysis revealed that the N-terminal half of ezrin shows homology to that of band 4.1 protein (~30% identity)(Conboy et al., 1986; Gould et al., 1989; Turunen et al., 1989). Recently, radixin was identified as a barbed-end capping actin-modulating protein from isolated cell-to-cell adherens junctions from the liver (Tsukita et al., 1989). Its cDNA sequencing revealed that radixin is highly homologous to ezrin (~75% identity for their full length; ~85% identity for their N-terminal half)(Funayama et al., 1991). Furthermore, moesin is also reportedly very similar to ezrin (~75% identity for their

full length; ~85% identity for their N-terminal half) (Lankes and Furth-
mayr, 1991; Sato et al., 1992). Moesin was originally identified as an
extracellular heparin-binding protein (Lankes et al., 1988) and was later
found to be an intracellular protein (Lankes and Furthmayr, 1991).   This
high homology between these proteins indicates a gene family (ERM family)
included in the band 4.1 superfamily (Sato et al., 1992). Recent detailed
immunolocalization analysis of these ERM family members revealed that they
are highly concentrated at the specialized domain of plasma membranes where
actin filaments are densely associated with plasma membranes, i.e. micro-
villi, cell-to-cell and cell-to-substrate adherens junctions, ruffling
membranes, and cleavage furrows. This suggests that these ERM family mem-
bers are directly involved in the actin filament/plasma membrane associa-
tion (Sato et al., 1992; Tsukita et al., 1992).

In addition to band 4.1 protein and ERM family members, talin was found
to belong to the band 4.1 superfamily (Rees et al., 1990).   Talin is a
protein with a molecular mass of ~200kD, which is concentrated at the
undercoat of cell-to-substrate adherens junctions (focal contacts) (Bur-
ridge and Connell, 1983; Burridge and Mangeat, 1984).   This protein is
thought to play an important role in linking integrins to actin-based
cytoskeletons. Recent cDNA cloning and sequence analysis revealed that the
domain homologous to the N-terminal half of band 4.1 protein exists in the
N-terminal end of talin molecules (Rees et al., 1990).   Furthermore, two
distinct protein tyrosine phosphatases called PTPH1 and PTPMEG are report-
edly members of band 4.1 superfamily, containing the band 4.1-like domain
and the phosphatase domain at their N- and C-terminal regions, respective-
ly (Gu et al., 1991; Yang et al., 1991).

Most recently, the tumor suppressor gene of neurofibromatosis 2 was

identified (Trofatter et al., 1993; Rouleau et al., 1993). Surprisingly, its gene product was very similar in amino acid sequence to the ERM family members (~49% identity for full length; ~62% for the N-terminal half). This product was named merlin (moesin-ezrin-radixin-like protein). Considering that in the case of band 4.1 protein the N-terminal half domain is responsible for its binding to glycophorin C and that this domain is homologous to the N-terminal regions of the band 4.1 superfamily members, all members are expected to be located just beneath the membrane through the specific binding of their N-terminal ends to integral membrane proteins. Furthermore, the fact that this superfamily includes protein tyrosine phosphatases and a tumor suppressor such as merlin as well as proteins responsible for the cytoskeleton/membrane interaction leads us to speculate that the band 4.1 superfamily members are directly involved in the regulation of cell growth by affecting the cytoskeleton/membrane interactions.

For a better understanding of the physiological functions of band 4.1 superfamily members, it is prerequisite to know how many proteins and what types of proteins are included in this superfamily. Therefore, in order to identify as many mouse band 4.1 superfamily members as possible, we used here polymerase chain reaction (PCR) and found several new members. In this study, we describe initial characterization of these new members and discuss the evolution of the band 4.1 gene superfamily.

Materials and Methods

Polymerase Chain Reaction (PCR)

Oligonucleotide primers were synthesized based on 6 conserved amino acid sequences described in detain in Fig.1. The degeneracy of primers A,B,C,D,E and F were 2^7-, 2^8-, 2^9-, 2^{10}-, and 2^9-fold, respectively. Phage DNA of a mouse embryonic teratocarcinoma F9 cell cDNA library (Nagafuchi et al., 1987), isolated by the plate lysate method (Sambrook et al., 1989), was used as template. Alternatively, a mRNA was prepared from F9 cells or mouse brain tissue by the use of a guanidium isothiocyanate method, and it was applied to the reverse transcriptase-PCR (RT-PCR). PCR conditions were essentially the same as those described by Saiki (1989). The PCR cycle, repeated at 30 times, consisted of denaturation at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 2 min. The PCR products were analyzed on a Nusieve GTG agarose (FMC Bioproducts, CO., Rockland, ME) and DNA fragments with reasonable sizes were excised, eluted from the gel, subcloned into the EcoRV site of pBluescript SK(-) plasmid (Stratagene), and sequenced.

Screening of the F9 Cell cDNA Library and DNA Sequencing

Two different λgt11 libraries made of mouse F9 poly(A) RNAs were used: In preparing these libraries, either a random mixture of hexanucleotides or oligo (dT) was used as primer for the first-strand synthesis (Nagafuchi et al., 1987). The PCR product of NBL4 was labeled with DIG according to the procedure developed by Boehringer Mannheim Biochemicals (Indianapolis,IN). Using this DIG-labeled fragment, ~5×10^6 plaques from the cDNA library was screened at high stringency, and one positive phage recombinant (α1) was

cloned (see Fig.3). Using two distinct fragments (Probes I and II in Fig.3) of α1 as probes, two more positive phage recombinants (β5, γ3) were cloned. All clones were subcloned into pBluescript SK(-) and sequenced with the 7-deaza Sequenase Version 2.0 kit (U.S.Biochemical Corp., Cleveland, OH), or with the Taq DyeDeoxy Terminator Cycle Sequencing Kit on an Applied Biosystems 373A Sequencer (Applied Biosystems, Inc. Foster City, CA).

Northern Blot Analysis

Total RNA was extracted from mouse various tissues and poly(A)$^+$ RNA was purified by oligo-dT-conjugated latex beads (Oligotex-dT30; Takara Shuzo Co., Ltd., Kyoto, Japan). 10μg of poly(A)$^+$ RNAs from each tissue was applied onto each well of a formaldehyde/agarose gel, separated electrophoretically, and transferred to a nitrocellulose membrane. An RNA ladder (Bethesda research laboratories, Bethesda, MD) was used as a size marker. Probes used were labeled with [α-^{32}P]dCTP using the Random Primer Labeling Kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Hybridization proceeded under conditions of high stringency (50% formamide/5xDenhardt's solution without BSA/5xSSC/0.5% SDS/100μg/m denatured salmon sperm DNA).

Results

Isolation of cDNAs encoding band 4.1 superfamily members

A PCR method was applied for the isolation of cDNAs that encode band 4.1 superfamily members. We carefully compared the amino acid sequences of the conserved N-terminal domain between all the band 4.1 superfamily members so far identified, chose six highly conserved amino acid sequences consisting of 6-8 amino acids, and synthesized the corresponding degenerate oligonucleotides (Fig.1). PCR and reverse transcriptase-PCR (RT-PCR) were performed with the mixed oligonucleotides as primers in every pair of the six conserved sequences. As templates, a mouse teratocarcinoma cell line (F9 cells) cDNA and the mouse brain mRNA were used, since the abundance of the band 4.1 superfamily members was expected in F9 cells and brain tissues. The resultant products showing the size appropriate for the combination of primers, were isolated and subcloned into the Bluescirpt SK(-) vector. In total, 216 clones with reasonable sized inserts were isolated (Table I). Sequence analysis of these clones revealed that 104 clones corresponded to ezrin, radixin, moesin, band 4.1 protein, PTPH1, PTPMEG, talin, or merlin, while the other 42 clones appeared to encode 6 new band 4.1 superfamily members. These new members are designated as NBL1~6 (Novel Band 4.1-like Proteins). Judging from their deduced amino acid sequences, these new members are subclassified into three types: Three (NBL1~3) and one (NBL6) of them showed a strong similarity to the corresponding sequence of PTPH1/ PTPMEG and merlin, respectively (Fig.2A,C). The other two members (NBL4,5) appeared to show no strong homology to the so far identified band 4.1 superfamily members (Fig.2B). Then, we attempted to isolated the full length cDNA encoding NBL4 from the λgt11

library made of mouse F9 cells.

Isolation and sequencing of cDNA encoding NBL4

To isolate the rest of the NBL4 gene, we screened ~5x10^6 plaques from a

λ gt11 cDNA library made from mouse F9 cells by DNA hybridization with the

DIG-labeled PCR product at high stringency, and cloned one positive phage

recombinant (α 1). Using two DIG-labeled fragments from α 1 (ProbeI&II in

Fig.3) as probes, two more positive phage recombinants (β 5, γ 3) were

cloned. Fig.3 shows the overall relationship of the PCR product, α 1, β 5,

and γ 3, and the restriction sites relevant for subcloning and sequencing.

The complete nucleotide sequence and deduced amino acid sequence of the

cloned molecule are shown in Fig.4. The composite cDNA is 2,503 nucleo-

tides long: 51 bp of the 5'-untranslated region, an ORF of 1,662 bp encod-

ing 554 amino acids, and 790 bp of the 3'-untranslated region with a

poly(A)$^+$ tail. A calculated molecular mass of the protein encoded by this

cloned cDNA is 61.0kD.

This cDNA has no long hydrophobic stretches which could encode a signal

peptide or a transmembrane domain, indicating that NBL4 is neither a secre-

tory protein nor a transmembrane protein. At the amino acid residue 2-7,

there is a typical myristoylation consensus (Towler and Gordon, 1988).

Furthermore, inside this molecule, several consensus sequences for possible

A-kinase- and tyrosine kinase-phosphorylation sites are found (Fig.4).

The N-terminal half of this molecule (amino acid residue 1-327) shows a

similarity to the N-terminal region of band 4.1 protein (39.9% identity),

ERM family members (29.2% identity), merlin (29.7% identity), PTPH1 (36.9%

identity), and talin (26.6% identity) (Fig.5). Considering that another

PCR product, NBL5, shows 95% identity to NBL4, this molecule together with

NBL4 appear to form a new family in the band 4.1 superfamily.   A search of the data bases identified no proteins with significant homology to the C-terminal half of NBL4.

To   detect mRNA encoding NFL4, Northern blot analysis with poly(A)$^+$ RNA obtained from various types of mouse tissues was performed. As   shown in Fig.6, the   band at 2.5 kb was detected in brain,   heart,   lung,   liver, spleen, and spleen, but undetactable in thymus and kidney.

Mouse merlin homologue and its gene family

We   have   noticed that the deduced amino acid sequence of one of   our   PCR products is almost identical to that of the corresponding region of human merlin (96% identity), suggesting that this PCR product is a part of the mouse homologue of merlin. Interestingly, we picked up another PCR product (NBL6)   which showed a strong similarity to mouse merlin (89% identity), suggesting that this protein together with merlin forms a gene family in the band 4.1 superfamily (see Fig.2C).

The   expression pattern of these merlin family members in various   types of tissues was   examined by Northern blot analysis. As   shown   in   Fig.7, mouse merlin was mainly expressed in brain and thymus, while the expression of NBL6 was restricted to brain.   Both Northern blot   analyses   revealed three hybridizing species of 2.5kb, 4.0kb and 5.5kb.

Discussion

Using the PCR method with mixed primers, we have attempted to clarify more detailed picture of the band 4.1 superfamily. So far, as band 4.1 super-family members, 8 proteins have been identified; band 4.1 protein, ezrin, radixin, moesin, merlin, talin, PTPH1, and PTPMEG (Tsukita et al., 1992). In this study, cDNA fragments have been obtained for all of these so far identified members, indicating that our screening system with the PCR method is very powerful to search for novel band 4.1 superfamily members.

Under such a screening system, in addition to cDNA clones for the so far identified members, we have picked up 6 cDNA clones which appear to encode novel band 4.1 superfamily members (NBL1~6). Sequence analyses of these clones revealed that three (NBL1~3) and one (NBL6) of them show a strong similarity to the corresponding sequence of PTPH1/PTPMEG and merlin, re-spectively, and that the other two members (NBL4,5) have no strong homology to the so far identified band 4.1 superfamily members. These data led us to conclude that the band 4.1 superfamily can be subclassified into at least 6 gene families; band 4.1 family (band 4.1 protein), ERM family (ezrin, radixin, moesin), talin family (talin), PTPH1 family (PTPH1, PTPMEG, NBL1, NBL2, NBL3), NBL4 family (NBL4, NBL5), and merlin family (merlin, NBL6). Among them, as to the band 4.1 and talin families, only one member was so far identified. Therefore, in a strict sense, they should not be called "family" at present. Of course, since the screening was performed using only mouse F9 cells and the brain in this study, it is possible that other families will be identified in different types of cells in future.

As shown in Fig.2A, PCR products of NBL1~3 show strong similarity to

the N-terminal half of PTPH1 and PTPMEG, pointing to the existence of a gene family, which is tentatively called PTPH1 family here. Recently, various types of protein-tyrosine-phosphatases were identified, and among them these PTPH1 and PTPMEG were characterized by the existence of the band 4.1 homology domain in their N-terminal half (Gu et al., 1991; Yang et al., 1991). These proteins contain the protein-tyrosine-phosphatase domain in their C-terminal half. At present, it is not clear whether or not NBL1～3 also have the protein-tyrosine-phosphatase domain. However, it is tempting to speculate that these novel members are also involved in the signal transduction just beneath the plasma membrane through some enzymatic activities such as phosphatase or kinase activities.

There remains to be no information about the functions of NBL4 family members, which were first identified in this study. The amino acid sequence of NBL4 deduced from its full-length cDNA has revealed that its N-terminal half has a similarity to band 4.1 superfamily members, but that its C-terminal half shows no homology with sequences in proteins so far identified. The clue to speculate the function of NBL4 was given only from the occurrence of consensus sequences for myristoylation, A-kinase-phosphorylation, and tyrosine kinase-phosphorylation. Further analysis with this NBL4 cDNA will lead us to a better understanding of functions of this novel protein.

The existence of the merlin family has not been so far recognized. One of our PCR products was almost identical (96% identity) to the corresponding part of human merlin, which is reportedly a candidate for the tumor suppressor of neurofibromatosis 2. This indicates that this cDNA fragment encodes a mouse homologue of merlin. Interestingly, another PCR product (NBL6) showed a strong similarity to this mouse merlin (89% identity),

pointing to the existence of the merlin family. Northern blot analysis revealed that both merlin and NBL6 are expressed preferably in brain. Considering that merlin plays a crucial role in the regulation of cell growth in the nervous tissue, it is likely that NBL6 is also involved in the cell growth regulation mainly in the nervous tissue. Further analyses on the interaction and relationship between merlin and NBL6 will be required to understand how merlin works as a tumor suppressor in neurofibromatosis 2.

In order to deduce the possible evolutionary relationship among the band 4.1 superfamily members, the amino acid sequences of the N-terminal homology of members were quantitatively compared, and the genetic distance for each pair of members was estimated according to the method described by Doolitle (1984). Based on these distances, a possible phylogenetic tree of the band 4.1 superfamily members was drawn in Fig.9 using the unweighed pair-group method (Fitch and Margoliash, 1967; Nei, 1987). The simplest interpretation of this tree is that talin and the primordial form of the other members were first generated and that the primordial form of the ERM/merlin families were differentiated from that of band 4.1/ NBL4/ PTPH1 families.

This study has clarified that various types of families are included in the band 4.1 superfamily, suggesting that the superfamily members are involved in various types of cellular events. However, it is likely that the band 4.1 homology domain localizing at the N-terminal domain of the members is responsible for their direct association with membrane proteins, suggesting that all the members work just beneath the plasma membrane. For a better understanding of the band 4.1 superfamily, we should next answer the question in what types of cellular events just beneath the

plasma membrane each family (in the band 4.1 superfamily) is involved.

References

Anderson,R.A. and R.E.Lovrien. 1984. Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. Nature 307:655-658.

Anderson,R.A. and V.T.Marchesi. 1985. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. Nature 318:295-298.

Bennett,V. 1989. The spectrin-actin junction of erythrocyte membrane skeletons. Biochim.Biophys.Acta 988:107-121.

Bretscher,A. 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. J.Cell Biol. 97:425-432.

Bretscher,A. 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. J.Cell Biol. 108:921-930.

Burridge,K. and L.Connell. 1983. A new protein of adherens plaques and ruffling membranes. J.Cell Biol. 97:359-367.

Burridge,K. and P.Mangeat. 1984. An interaction between vinculin and talin. Nature 308:744-746.

Conboy,J., Y.W.Kan, S.B.Shohet, and N.Mohandas. 1986. Molecular cloning of protein 4.1, a major structural element of the human erythrocyte membrane skeleton. Proc.Natl.Acad.Sci.USA 83:9512-9516.

Doolitle,R.F. 1984. In Putnam,F.W. (ed.), The Plasma Proteins. Academic Press., Orlando,FL, Vol.IV, pp.317-360.

Fitch,W.M., and E.Margoliash. 1967, Construction of phylogenetic trees. Science, 155:279-284.

Fowler,V. and D.L.Taylor. 1980. Spectrin plus band 4.1 cross-link actin. J.Cell Biol. 85:361-376.

Funayama,N., A.Nagafuchi, N.Sato, Sa.Tsukita, and Sh.Tsukita. 1991. Radixin is a novel member of the band 4.1 family. J.Cell Biol. 115:1039-1048.

Gould,K.L., A.Bretcher, F.S.Esch, and T.Hunter. 1989. cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. EMBO J. 8:4133-4142.

Gould,K.L., J.A.Cooper, A.Bretscher and T.Hunter. 1986. The protein-tyrosine kinase substrate, p81, is homologous to a chicken microvillar core protein. J.Cell Biol. 102:660-669.

Gu,M., J.D.York, I.Warshawsky, and P.W.Majerus. 1991. Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. Proc.Natl.Acad.Sci.USA 88:5867-5871.

Hunter,T. and J.A.Cooper. 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell 24:741-752.

Hunter,T. and J.A.Cooper. 1983. Role of tyrosine phosphorylation in malignant transformation by viruses and in cellular growth control. Prog.Nucleic Acid Res.Mol.Biol. 29:221-233.

Lankes,W., and H.Farthmayr. 1991. Moesin: A member of the protein 4.1-talin-ezrin family of proteins. Proc.Natl.Acad.Sci.USA 88:8297-8301.

Lankes,W., A.Griesmacher, J.Grunwald, R.Schwartz-Albiez, and R.Keller. 1988. A heparin-binding protein involved in inhibition of smooth-muscle cell proliferation. Biochem.J. 251:831-842.

Leto,T.L., I.Correas, T.Tobe, R.A.Anderson, and W.C.Horne. 1986. In Membrane Skeleton and Cytoskeletal Membrane Associations. V.Bennet, C.M.Cohen, S.E.Lux, and J.Palek, eds. Alan R.Liss, New York. 201-209,

Mueller,T.J. and M.Morrison. 1981. In Erythrocyte Membranes 2: Recent Clinical and Experimental Advances. Kruckeberg, Eaton and Brewer, eds. Alan R.Liss, New York. 95-112.

Nagafuchi,A., Y.Shirayoshi, K.Okazaki, K.Yasuda, and M.Takeichi. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. Nature 329:341-343.

Nei,M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, N.Y. p.293-298.

Ohanian,V, L.C.Wolfe, K.M.John, J.C.Pinder, S.E.Lux, and W.B.Gratzer. 1984. Analysis of the ternary interaction of the red cell membrane skeletal proteins spectrin, actin, and 4.1. Biochemistry 23:4416-4420.

Pakkanen,R., K.Hedman, O.Turunen, T.Wahlstrom, and Vaheri,A. 1987. Microvillus-specific Mr 75,000 plasma membrane protein of human choriocarcinoma cells. J.Histochem.Cytochem. 135:809-816.

Rees,D.J.G., S.E.Ades, S.J.Singer, and R.O.Hynes. 1990. Sequence and domain structure of talin. Nature 347:685-689.

Rouleau,G.A., P.Merel, M.Lutchman, M.Sanson, J.Zucman, C.Marineau, K.Hoang-Xuan, S.Demczuk, C.Desmaze, B.Plougastel, S.M.Pulst, G.Lenoir, E.Bijl-sma, R.Fashold, J.Dumanski, P.de Jong, D.Parry, R.Eldrige, A.Aurias, O.Delattre, and G.Thomas. 1993. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 363:515-521.

Saiki,R.K. 1989. PCR Technology (H.A.Erlich, ed.), Stockton Press,N.Y. p.7-22.

Sambrook,J., E.F.Fritsch, and T.Maniatis. 1989. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab., Cold Spring Harbor,NY.) 2nd Ed.

Sato,N., N.Funayama, A.Nagafuchi, S.Yonemura, Sa.Tsukita, and Sh.Tsukita. 1992. A gene family consisting of ezrin, radixin, and moesin. Its specific localization at actin filament/plasma membrane association sites. J.Cell Sci. 103:131-143.

Towler,D.A., and J.I.Gordon. 1988. The biology and enzymology of eukaryotic protein acylation. Ann.Rev.Biochem. 57:69-99.

Tsukita,Sa., Y.Hieda and Sh.Tsukita. 1989a. A new 82 kD-barbed end capping protein localized in the cell-to-cell adherens junction: Purification and characterization. J.Cell Biol. 108:2369-2382.

Tsukita,Sh., Sa.Tsukita, A.Nagafuchi, and S.Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell-to-cell adherens junctions. Curr.Opin.Cell Biol. 4:834-839.

Trofatter,J.A., M.M.MacCollin, J.L.Rutter, J.R.Murrell, M.P.Duyao, D.M.Parry, R.Eldridge, N.Kley, A.G.Menon, K.Pulaski, V.H.Haase, C.M.Ambrose, D.Munroe, C.Bove, J.L.Haines, R.L.Martuza, M.E.MacDonald, B.R.Seizinger, M.P.Short, A.J.Buckler, and J.F.Gusella. 1933. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 72:791-800.

Turunen,O., R.Winqvist, R.Pakkanen, K.H.Grzeschik, T.Wahlstrom, and A.Vaheri. 1989. Cytovillin, a microvillar Mr 75,000 protein. cDNA sequence, prokaryotic expression, and chromosomal localization. J.Biol.Chem. 264:16727-16732.

Ungewickell,E., P.M.Bennett, R.Calvert, V.Ohanian, and W.Gratzer. 1979. In vitro formation of a complex between cytoskeletal proteins of the human erythrocyte.   Nature 280:811-814.

Yang,Q. and N.K.Tonks. 1991. Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associated proteins band 4.1, ezrin, and talin.   Proc.Natl.Acad.Sci.USA 88:5949-5953.

Band 4.1 homology domain (370 a.a.)

A:EK(E/D/W)YFGL   38-44aa   D:(K/N)ISF(K/N)R   232-237aa

B:LAS(Y/F)AVQ   119-125aa   E:RK(K/Q/S)F(F/V)I   237-242aa

C:AR(T/D)L(D/F)(F/L/M)YG 181-188aa   F:CVEHHTFF or CMGNHELY 271-277aa
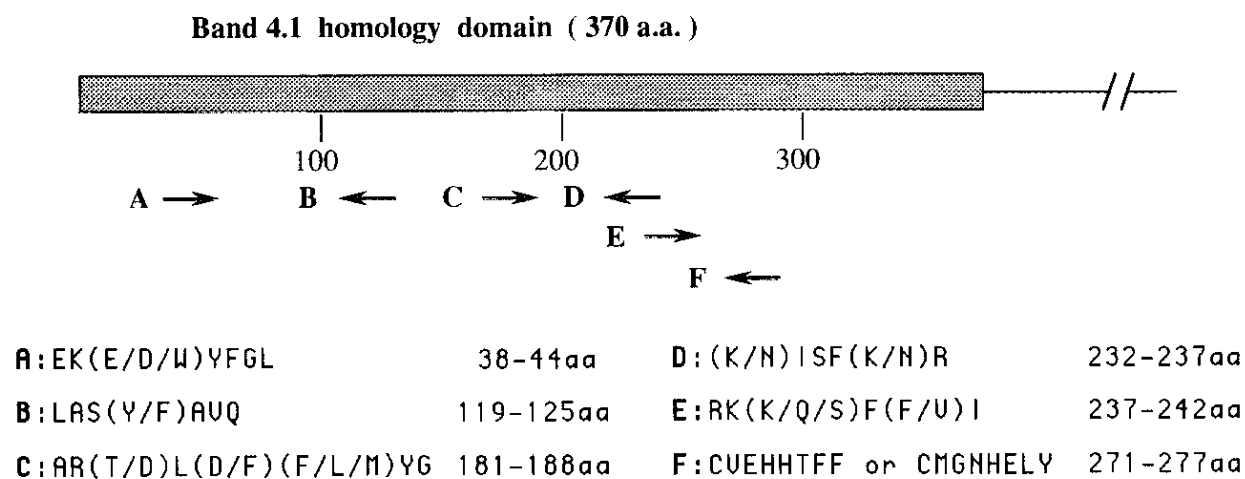
Figure 1. Highly-conserved amino acid sequences among so far identified band 4.1 superfamily members. For the PCR analysis, 6 sequences (A-F) were chosen in the conserved domain (~370 a.a.) among band 4.1 superfamily members. Corresponding degenerate oligonucleotides were synthesized in one direction.

Table 1.   Number of independent cDNA subclones

	No. of clones sequenced	Band4.1	Ezrin	Radixin	Moesin	Talin	PTPH1	PTPMEG	NBL1	NBL2	NBL3	NBL4	NBL5	Merlin	NBL6
	20	---	2	4	3	---	4	2	2	---	1	2	---	---	---
	112	5	17	12	21	---	16	15	6	4	2	10	4	---	---
	20	---	4	5	6	---	---	2	---	---	---	3	---	---	---
	41	3	8	6	8	---	2	2	3	---	2	---	---	5	2
	9	---	2	---	2	---	3	1	1	---	---	---	---	---	---
	14	2	2	1	1	3	4	---	---	---	---	1	---	---	---

PCR was carried out using primers shown in the left diagram of the band 4.1 homolog domain (open box) with mouse F9 cells and brain cDNA templates. The products were size-fractionated by agarose gel electrophoresis, eluted, subcloned, and sequenced.

(A)

```
              *   *    *                                                **       *    *    *      **
Band4.1    VDLHKAKDLEGVDIILGVCSSGLLVYKDKLRINRF-PWPKVL-KISVKASSFFIKIRPGEQ-EQVESTIGFKLPSYRAAKKLWKV
PTPH1      VELHGGRDLDNLDLMIGIASAGIAVCRKYICTSFV-PWVNIL-KISFKRKKFFIHQRQKQQAESAEHIVAFNMLNVRSCKNLWKS
PTPMEG     VEFHVAADQSNNEILIGVMSGGILIVKNR-VRMHTFLWLKIV-KISFKCKQFFIQLR-KELHESRETLLGFNMVHVRACKTLWKA
NBL1       VELHKGRDLDQLHLMIGVASGGILVCRKR-VRTHRFPWPNILUKISFKRKKFFIQQRQKEAEE-RESTVGFNMLHVRACKNLWKS
NBL2       VELHKARDLDQLHGEHKSAVGGILTPRKRVVRTNKKPWGKIF-WPRIKRVHFFITQFEKEVLEK-ESTETFNFLHARASCTLWKS
NBL3       VEFHKARDLSQNEIENGSMSGLGLTPRKRVRMNNKKPKLIIK-WKISFKRKKFITQRQKEVLSR-ESTETFNFLHVRRSCKKWKS
```

(B)

```
           ****************** '******** ' **** ' ********** ' ***************************************
NBL4       VDLHPUVGENKSEVFLGLTPSGVUUVYKNKKQVGKVFWPRITKVHFKETQFELRVLGKDCNETSFFFEARSKTRCKHLWKC
NBL5       VDLHPUVGENKSEVFLGVTPSGVUUVYKRKKQVEKVFWPRITKIHFKETQFELRVLGKDCNETSFFFEARSKTRCKHLWKC
```

(C)

```
           ************************* '*** ' ' '********** ' ***
Merlin     VNVFRIRNKKGTELWLGVDRLGLHIVDPENRLTPKISFPWSEIR
NBL6       VNVFRIRNKKGTELWLGVDRLGLNIVDQKDRLTPKISFPWNEIR
```

Figure 2. Alignment of PTPH1 family member sequences (A), NBL4 family member sequences (B), and merlin family member sequences (C). (A) Comparison of deduced amino acid sequences of the PCR product of band 4.1 protein with those of the PTPH1 family members. Conserved amino acids shared by more than 5 proteins are shadowed. The positions where all sequences share an identical amino acid are indicated by asterisks. Gaps, introduced to maximize alignment, are indicated by dashes. (B,C) Amino acid sequences of PCR products of NBL4 family members (B) and merlin family members (C). Identities are indicated by asterisks. These are not artifacts derived from the PCR method, since at the nucleotide sequence level the identity for NBL4/NBL5 and merlin/NBL6 are 72% and 68%, respectively.
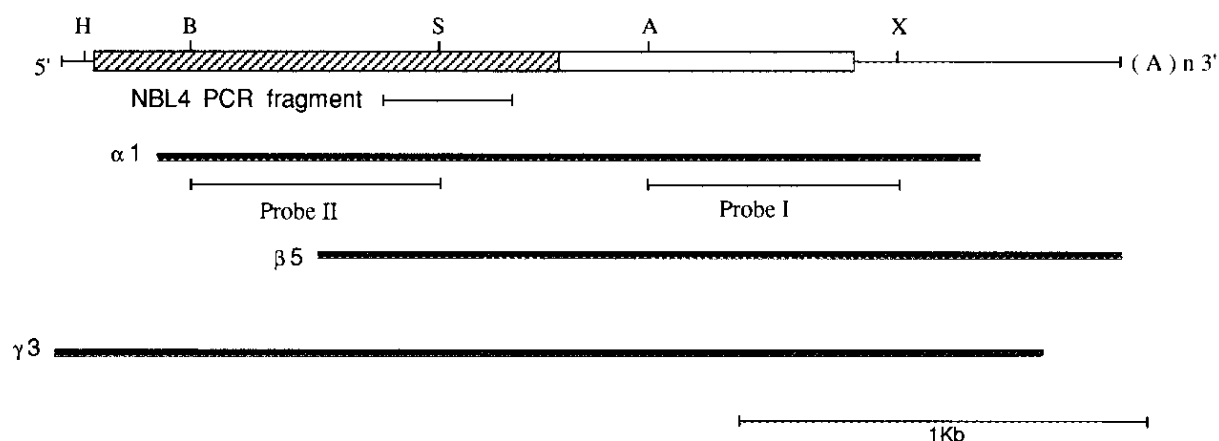
Figure 3. Restriction map and cDNA fragments of NBL4. The box indicates the coding region, and the band 4.1 homology region is shadowed. α1 was first obtained from cDNA library made from mouse F9 cells using the PCR product (NBL4 PCR fragment) as a probe. β5 and γ3 were obtained using Probe I and Probe II as a probe, respectively. A,AvaI; B,BamHI; H,HindIII; S,SacI; X,XbaI.

```
   1  GCGGGCGCCGCGGTCGCAGCTCTCGCCTTGCTGGAGAAGCTTTCAGCCGACATGGGCTGTTTCTGCGCCGTTCCGGAAGAATTTTACTGCGAAGTTTTGCTCCTGGACGAGTCCAAGCTC
                                                M  G  C  F  C  A  V  P  E  E  F  V  C  E  V  L  L  L  D  E  S  K  L

 121  ACCCTCACCACCCAGCAGCAGGGCATCAAGAAGTCAACAAAAGGCTCTGTTGTCCTTGACCACGTTTTCCGTCACATAAACCTCGTGGAGATAGATTATTTTGGGCTGCGGTACTGTGAC
         T  L  T  T  Q  Q  Q  G  I  K  K  S  T  K  G  S  V  V  L  D  H  V  F  R  H  I  N  L  V  E  I  D  Y  F  G  L  R  V  C  D

 241  AGAAGCCATCAGACATATTGGCTGGATCCCGCAAAAACCCTTGCAGAACACAAGGAGCTGATCAACACTGGACCTCCATATACTTTGTATTTTGGTATTAAATTCTATGCTGAAGATCCA
         R  S  H  Q  T  Y  W  L  D  P  A  K  T  L  A  E  H  K  E  L  I  N  T  G  P  P  Y  T  L  Y  F  G  I  K  F  Y  A  E  D  P

 361  TGTAAACTCAAAGAAGAAATAACCAGATATCAGTTTTTCTTACAGGTGAAGCAAGATGCCCTTCAGGGCCGGCTGCCTTGCCCTGTCAATATTGCTGCTCAGATGGGGGCATATGCCATC
         C  K  L  K  E  E  I  T  R  Y  Q  F  F  L  Q  V  K  Q  D  A  L  Q  G  R  L  P  C  P  V  N  I  A  A  Q  M  G  A  Y  A  I

 481  CAGGCCGAACTGGGAGACCACGACCCATACAAGCACACTGCCGGGGTATGTGTCGGAGTACCGGTTTGTTCCTGATCAGAAGGAAGAGCTGGAAGAAGCCATAGAAAGGATTCATAAAACT
         Q  A  E  L  G  D  H  D  P  Y  K  H  T  A  G  V  V  S  E  Y  R  F  V  P  D  Q  K  E  E  L  E  E  A  I  E  R  I  H  K  T

 601  CTAATGGGTCAGGCTCCTTCCGAAGCTGAGCTGAATTACTTGAGGACTGCCAAATCCCTGGAGATGTATGGTGTTGACCTCCATCCTGTCTATGGAGAAAATAAGTCCGAGTACTTCTTA
         L  M  G  Q  A  P  S  E  A  E  L  N  Y  L  R  T  A  K  S  L  E  M  Y  G  V  D  L  H  P  V  Y  G  E  N  K  S  E  Y  F  L

 721  GGCTTGACTCCATCGGGAGTTGTCGTCTATAAGAATAAAAAGCAAGTGGGGAAGTATTTCTGGCCTCGGATTACAAAGGTGCACTTCAAGGAAACCCAGTTTGAGCTCAGAGTCTTGGGA
         G  L  T  P  S  G  V  V  V  Y  K  N  K  K  Q  V  G  K  Y  F  W  P  R  I  T  K  V  H  F  K  E  T  Q  F  E  L  R  V  L  G

 841  AAAGACTGTAATGAAACCTCATTCTTTTTTGAAGCTCGAAGCAAAACTGCTTGCAAGCACCTCTGGAAATGCAGCGTAGAGCACCATACGTTTTTCAGAATGCCGGACACAGAATCAAAT
         K  D  C  N  E  T  S  F  F  F  E  A  R  S  K  T  A  C  K  H  L  W  K  C  S  V  E  H  H  T  F  F  R  M  P  D  T  E  S  N

 961  TCATTATCAAGAAAACTCAGCAAGTTTGGGTCCATAAGTTATAAACATCGGTACAGGACAGCTTTGCAAATGAGCCGAGATCTTTCTATCCAACTTCCCCGGCCCAATCAGAACGTGGTG
         S  L  S  R  K  L  S  K  F  G  S  I  S  Y  K  H  R  Y  R  T  A  L  Q  M  S  R  D  L  S  I  Q  L  P  R  P  N  Q  N  V  V

1081  AGAAGTCGAAGCAAGACTTACCCCAAGAGGGTAGCACAGACTCAGCCCACTGGATCAAACAACATCAATCGGATAACTGCAAACACGAAAACGGGGAGAATGAAGGAACCACCAAGATC
         R  S  R  S  K  T  Y  P  K  R  V  A  Q  T  Q  P  T  G  S  N  N  I  N  R  I  T  A  N  T  E  N  G  E  N  E  G  T  T  K  I

1201  ATTGCGCCTTCCCCAGTGAAAAGTTTTAAGAAAGCAAAGAATGAAAACAGCCCTGACCCCCAAAGAAGCAAATCTCATGCACCTTGGGAAGAAAACGGACCGCAGAGTGGACTATACAAC
         I  A  P  S  P  V  K  S  F  K  K  A  K  N  E  N  S  P  D  P  Q  R  S  K  S  H  A  P  W  E  E  N  G  P  Q  S  G  L  Y  N

1321  TCCTCCAGTGACCGCACTAAGTCACCAAAGTTTCCCTGTGCCCGGCAGCGCAACCTCTCCTGCGGCAGCGACAATGACTCCTCACAACTGATGAGGCGAAGGAAAGCTCACAACAGTGGC
         S  S  S  D  R  T  K  S  P  K  F  P  C  A  R  Q  R  N  L  S  C  G  S  D  N  D  S  S  Q  L  M  R  R  R  K  A  H  N  S  G

1441  GAAGACTCAGATCTCAAGCAGAGGAGGAGGTCACGCTCACGCTGTAACACAAGCAGTGGTAGTGAGTCAGAAAATTCTAACAGAGAACACCGGAAAAAGAGAAACAGAACCCGCCAGGAG
         E  D  S  D  L  K  Q  R  R  R  S  R  S  R  C  N  T  S  S  G  S  E  S  E  N  S  N  R  E  H  R  K  K  R  N  R  T  R  Q  E

1561  AATGACATGGTTGACTCGGGGCCTCAGTGGGAAGCCGTGTTAAGGAGCACAGAAGGAAAAGAACCAAGCCGGACCCTAACAACCGGAGATCCAGACACAGATCTCGCTCAAGAAGTCCTGAC
         N  D  M  V  D  S  G  P  Q  W  E  A  V  L  R  R  Q  K  E  K  N  Q  A  D  P  N  N  R  R  S  R  H  R  S  R  S  R  S  P  D

1681  ATCCAAGCAAAAGAAGAACTGTGGAAGCACATCTAGAAGGAGCTTGTGGACCCATCTGGACTGTCAGAAGAGCAGCTGAAGGAGATCCCGTACACTAAAGTAGAGACTCAGGGTGACCCG
         I  Q  A  K  E  E  L  W  K  H  I  *

1801  GTCCGCATCCGGCATTCTCATTCCCCGCGGAGTTACCGGCAGTACCGAAGATCCCAGTGTTCAGACGGAGAACGATCCGTTCTCTCGGAAGTGAACTCAAAGACAGATCTTGTACCACCA
1921  CTTCCGGTCACCCGTTCCTCAGATGCTCAGGGGTCTGGAGGCTCGACAGTTCATCAGAGGAGAAATGGGTCTAAAGACAGCTTGATTGAAGAAAAGTCTCAGCTATCTACAATCAACCCG
2041  GCTGGGAAACCTACAGCAAAAACCATAAAAACCATCCAGGCTGCTCGCCTCAAGGCAGAGACTTGACGGGCTGGGTGCGGGGTAGGAAGGGAAATTCGTGCCGTGCCACTGGTACTTCTG
2161  AAACTGTGAATTAGGTCATTATCCATGCACCCTGGACATGTGTCTTTAGCATCGTCTAAGGATTTATGTACAGCATTCTTGTGCTCTGGGCCTTTGGGAGGACAACCTTATAAACTCACT
2281  GGATACCACCATCTATTTCCTGAAATTCTGACCATCCAACTCTCCACCCAGTCAAGACCAGCAACTTCCGATGAGCCCCTGGACTCTGGGCCTGCATCAGACTGGTGACTTTTATTCCCA
2401  CCAAACTGTTTCTAATATTGTATCCCGTGGCACTAAGTCCCAACGGTTATCTACAAATAATGTAAAAAGCTGTTAAGTGTTGATTTGAAAAAAAAAAAAAAAA
```

Figure 4. Nucleotide and deduced amino acid sequences of NBL4. The ORF encodes a 554 amino acid polypeptide with a predicted molecular mass of 61.0kD. The band 4.1 homology domain is shadowed. The consensus sequences for myristoylation (═══), A-kinase phosphorylation (-----), and tyrosine-kinase phosphorylation (━━━) are underlined.

```
MERLIN     M-SFSSLKR KQ-------- PKTFT----- VRIVTMDAEM -EFNCEMKWK KDLFD CR TLGLR WF GL Y-TIK-D TVA KMI K   72
RADIXIN    M--------- --------- PKPIN----- VRVTTMDAEL -EFAIQPNTT KQLFD V K TVGLR WF GL YVDSK-G YST WI K LNK  64
BAND41     MH-------- ---------- CKVSLLDDTV YECVVEKHAK DDLLKR CE HLNLI DY GL AIWDNA-T SKT WI DSAK  61
PTPH1      MTSRLRALGG RINNIRTSEL PKEKTRSEVI CSIHFLDGVV QTFKVTKQDT GDVLLDM N HLGVI KEY GL HDDDDSVD SPR WL EASK  90
NBL 4      MGC------- ------FCAV PEEFYCEVLL LDESKLTLTT QQQGIKKSTK GBVVLDH FR HINLVE DY GL RYCDRS-H QTY WL DPAK  76

Consensus  M......... ....----- PK...----- .....LD... .EF...K..K .LLD.. . HLGL.. . YE GL Y.D... .. ...WI..  90


MERLIN     VL-DHDV-SK ER VTFH LA H YPENAEEE VQEI DHLF H QVKKQILD EKIY PPEAS VLLAS AV A KY GD DPSV KRGFLAQEEL  160
RADIXIN    VT-QQDV-KK ENP LQFK FRA H FPEDVSEE LQEI DRLF H QVKEAILN DEIY PPETA VLLAS AV QA KY GD YNKEI H KPGYLANDRL  152
BAND41     I--KKQV-R- GVP WNFT FNV H FPPDPA-Q LTEDI RYYL Q LRQDIVA GRLR CFATL ALLGS YI OS EL GD YDPEI H GVDYVSDFKL  146
PTPH1      I--RKQL-KG GR CTLH FRV H FIPDPN-T LQEC RHLY H LKMDICE GRLT CPLNSA VVLAS YA OS HR GD YNSSI H HPGYLSDSHF  176
NBL 4      LAEHKEI INT GF YTLM FG I K FYAEDPC-K LKEET RYQF H LQVKQDALQ GRLR CPVNIA AQMGA YA A EL GD HDPYK H TAGYVSEYRF  165

Consensus  ..-.K.V-.. G .. TF E.. K YPEDP.-. LQEI R.LF H QVK.DIL. GRL. CP...A VLLAS AV Q. ..GD DP..H .GYI S...L  180


MERLIN     I KRVINLYQ MTPEMWEERI TAWYAEHRGR ARDEAEMEYL K AD EMYG V NYFAIRNKK GTELL G DA I LHIT DPEN RLTPKISFPW  250
RADIXIN    I PORVLEQHK LTKEQWEERI QNWHHEEHRCM LREDSMMEYL K AD LSMYG V NYFEIKNKK GTELW G DA I G LNIM EHDD KLTPKIGFPW  242
BAND41     AP NQTKELEE -------KV MELHKSYRSM TPAQADLEFL EN AK LSMYG V DLHKAKDLE GVDJI IL G CS SO LIA YKDKL RINRFPWPKV  228
PTPH1      IP QNEDFLT --------KV ESIHEQHSGI KQSEAESCYI NI ARTI DHYG V ELHSGRDLH NLDLMI GIAS AG VA VYRKYI CTSFYPWVNI  258
NBL 4      VP QKEELEE -------AI ERIHKTIMGQ APSFAELNYI RT AKS LEMYG V DLHPVYGEN KSEYFI G LTP SG VVY KNKK QVGKYFWPRI  247

Consensus  I.Q..EL.. .--------I ...H..HRG. ...FAE.EYL .I A. EMYG V LH...... G.EL.IG V.. I.L.VY. ......W...  270


MERLIN     NEIRNISYSD KEFTIKPLD KKIDVFK NS SKLRVN KLIL QLCIGN FDLF M- -RRRKADS LEVQQMKAQA RE------E KA----RKEM  326
RADIXIN    SEIRNISFND -KKFVIKP1D KKAPDFV FYA PRLRIN KRIL ALCMGN HELY M--RRRKPDT IEVQQMKAQA RE------V LH----QKQL  318
BAND41     LKISYKRSSF FIK1RPGFQE QYESTIG FKL PSYRAA KKIW KVCVFH HFF R---LTSTDT 1PKSKFLALG SK--------I RY-----SG  302
PTPH1      LKISFKRKKF FIHQRQKQAE SREH1VA FNM LNYRSO KNLW KSCVEH IFF QAKKLLPQFK NVLSQYWTMC SRNTKKSVNN QYCKKV1GGM  348
NBL 4      TKVHFKFTQF --ELRVLGKD CNETSFF FEA RSKTAO KHLW KCSVEH HFF R---MPDTES NSI.SRKLSKF GS- ------I SY-----KHR  320

Consensus  .KI..K...F -...R.K..D ..E..F H. ...R. KI LW K.CVEH FF ...-...D. ...SQ..A.. ......... .Y-----K..  360


MERLIN     ERQRLAREK -----QMRFE AERTRD--- ------ELE FRLI QMKEEA T-------- ----- --MA NEALMRSE-- ----FTAD-  374
RADIXIN    ERAQLENEK -----KKREI AEKEKE--- ------R1E REKEELMERL R-------- ----- -QI EEQTVKAQ-- --KFLE--  366
BAND41     -RTQAQTR-- ----QASAL IDRPAP---- -- ---HFE R ASKRASRS L-------- ----- DG AAAVDSAD-- - RSPR--  348
PTPH1      VWNPAMRRSL SVEHLETKSL PSRSPP1TPN WRSPRLRHE1 RKPRHSSADN LANEMTYITE TEDVFYTYKG SLAPQDSDSE VSQNRSPHQE  438
NBL 4      YRTALQMSR- --- DLSIQ LPRPNQ-- ----- NVV RGRSKTYPKR V--------- -- ------A QTQPTGSN-- ---NINR--  367

Consensus  .R..I..... --- .... R.... ----- ..E R ........ ......... ......... ..A...S. ........  450


MERLIN     --------- --------- -LLAEKA-- ---- --- QITEFE AKLLA-- --- ----- ---- ----QKA AE----- -  396
RADIXIN    --------- ------ -EQTRKA-- --- ----- ---- LELEQ ERQRA---- ----- ---- ---- -KEF AE -----  387
BAND41     --------- -------- -PTS-APA ----- ----- ----- ----- ITQGQ VAEGG ----- ----- VL---- DAS AK---- -  371
PTPH1      SLSENNPAQS YLTQKSSSSV SPSSNAPGSC SPDGVDQQLL DDFHRVTKGG STEDASQYYC DKNDNGDSYL VIJRITPDED GKFGENLKGG  528
NBL 4      --------- --- --- ----- --- --- ---- ----- IT--A NTENG---- ----- --- -- ---ENE GT------  380

Consensus  --------- --- ---- ..... .... ---- ---- ----IT... ..E.A---- - ----- ----- ... A. ----  540


MERLIN     --------- ---- --AEQ EMQ--- --- -------- ----- ---- -RIKAT---- ------ -A IRTEEEKRLM EQ ------  420
RADIXIN    --------- --------RI DRE ------ -------- --------- --------- -RRAAE---- ------E AKSATAKQAA DO---- ---  410
BAND41     --------- ------KT VVP------ -------- --- ----- KAQKE---- ------ --T VK- AFVKKE DE- -----  392
PTPH1      VDQKMPLVVS RINPESPADT C1PKLNEGDQ IVLINGRDIS EHTHDQVVMF IKASRESHSR ELALVIRRRA VRSFADFKSE DELNQLFPFA  618
NBL 4      --------- --------TK II- - ---- -------- -- ----- ----- -APS---- ----- ---P VKSFKKAKNE NS----  400

Consensus  --------- ------- .. ---- ---- ------ ------ -.A..E---- - ----- . VKS...K.E D..  630


MERLIN     ----KVLEAE VLALKMAEES ERRAK- ---- -EADQLKQDL Q-------- EAREA---- --------- ---- -ERR AKQKL----L  465
RADIXIN    -----MKNQE QLAAELAEFT AKIAL----- -1EEAKKKKE E------ EATEW----- ------ --- QHK AFAAQ ---F  454
BAND41     --P--PEQAE P--EPTEAWK KKRER----L DGENIYIRHS NL------ML EDLDK----- --------- --SQEE-IKK HHASI----S  444
PTPH1      IFPMCPEGGD TLEGSMAQLK KGLESGTVLI QFEQLYRKKP GLAITFAKLP QNLDKNRYKD VLPYDYTRVL LQGNEDY1NA SYVNMEIPAA  708
NBL 4      -----PDPQR S--KSHAPWE ENGPQ----- --SGLYNSSS ---------- ---DR---- ----- ---TKS P- -------

Consensus  -----P...E ...P....-- ----.D...S .......... .G........- ----...... E..D.---- --------- ........  720


MERLIN     EIATKPTYP- PMNPIPAP-- ----LPPDIP SFNLIGDSLS FDFKDTD--- ------MKRL SMEIEKEKVE YMEKSKHLQE QLNELKT---  536
RADIXIN    DL-EKTKEE- LKTVMSAP-- ----PPPPPP PVIPPTEN-E HDEQDEN--- ------SAEA SAELSSEGV- -MNHRSEEEW VTETQKN---  521
BAND41     ELKKNFMES- VPEPRPSE-- ----WDKRLS THSPFRTLNI NGQIPTGE-- -GPP-LVKTQ TVTISDNANA VKSEIPTKDV PIVHTET---  520
PTPH1      NLVNKYIATQ GPLPHTCAQF WQVVWDQKLS LIVMLTTLTE RGRTKCHQYW PDPPDVMNHG GFHIQCQSED CTIAYVSREM LVTNTQTGEE  798
NBL 4      ----KFPCA- RQRNLSCG-- ----SDNDSS QLMRRRKAHN SGEDSDLK-- ------QRRR SRSRCNTSSG SESENSNREH RKKRNRT---  500

Consensus  .L..K....- ...P....-- -----.D...S .......... .G........- ----....... S..I...... ........E. ......T---  810


MERLIN     EIE------- --ALKLKER- --------- ETALAILHNE NSDRGGSSKH NTIK------ ---------- ---KLTLQSA KSRVAFFEE-  586
RADIXIN    ERV------- --KKQLQAL- -------- SSELAQARDE TKKTQNDVLH AENV----- ---------- ---KAGRDKY KTLRQIRQG-  571
BAND41     KTIT---YEA --AQTVKGG- ---------I SETRIE-KRI VITGDADIDH DQVLV----- ---------- ---QAIKEAK EQHPDMSVT-  575
PTPH1      HTVTHLQYVA WPDHGIPDDS SDFLEFVNYV RSLRVDSEPV LVHCSAGIGR TGVLVTMETA MCLTERNLPI YPLD1VRKMR DQRAMMVQTS  888
NBL 4      RQ------- --ENDMVDS- ---------- GPQW---EAV LRR-QKEKNQ AD------- ---------- ---PNNRRSR HRSRSRSPD-  543

Consensus  ...------- --.,...... ---------- .......... ..........H ...-...... .... ----- . ...R... ..........-  900


MERLIN     --LQV----- --TQPPQDL- ---PLL                                                                        599
RADIXIN    ---NT----- --KHAIDEF- --EAM                                                                         583
BAND41     ---KVV---- --VHQETEI- ---ADE                                                                        588
PTPH1      SQYKFVCEAI LRVYEEGLVQ MLDPSN                                                                         914
NBL 4      --------- --IQAKEEL -WKHI-                                                                          555

Consensus  --,.,----- --,...E.- ---...                                                                          926
```

Figure 5. Alignment of the deduced amino acid sequences of five band 4.1 superfamily members whose full length cDNAs were obtained. Conserved amino acids are indicated in "consensus". The positions where all sequences share an identical amino acid are boxed. Gaps, introduced to maximize alignment, are indicated by dashes. Note that the amino acid sequences are highly conserved at their N-terminal half domains.
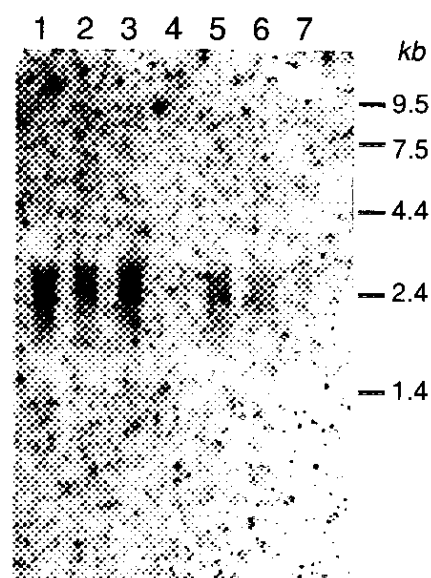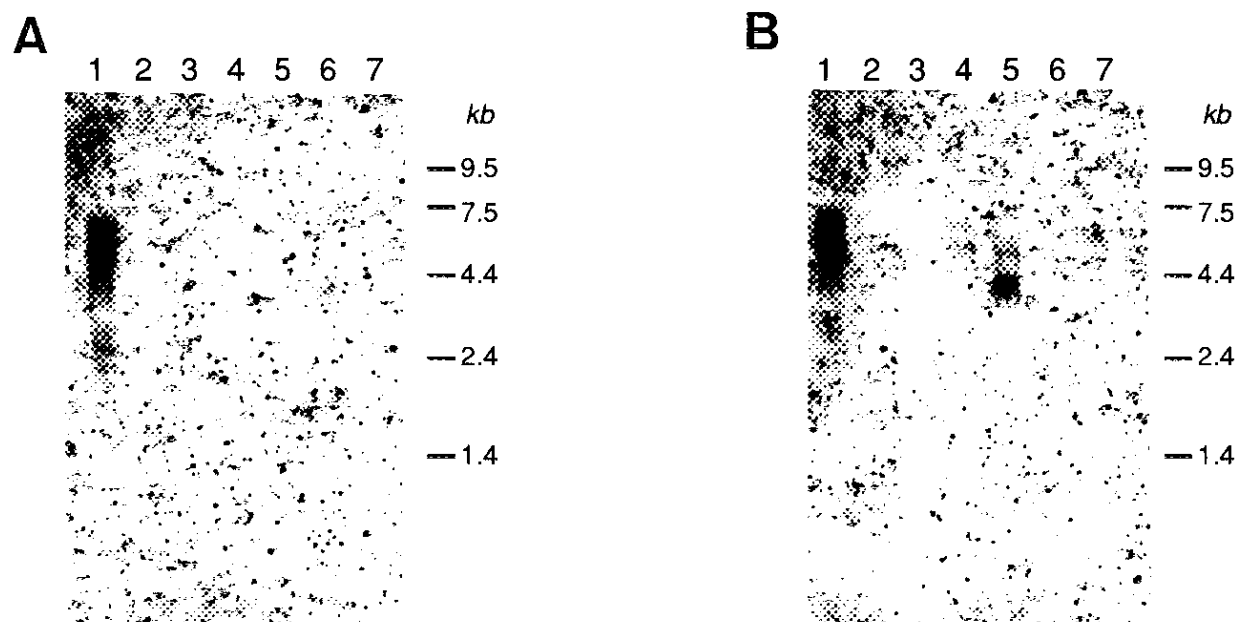
Figure 6.   Detection of NBL4 mRNA in various types of tissues by Northern blot hybridization.   The poly(A)$^+$ RNA samples were electrophoresed in agarose gel and transferred to a nitrocellulose filter.   The HindIII-AvaI fragment of NBL4 (see Fig.3) cDNA was used to probe the RNA blot. Mobility of RNA standards (kb) is shown at right.   Lane 1, brain;   lane 2, heart;   lane 3, lung; lane 4, thymus; lane 5, liver; lane 6, spleen; lane 7, kidney.   Note that the expression level of NBL4 is rather high at brain, heart and lung.

A

B

Figure 7.   Detection   of NBL6 (<u>A</u>) and merlin (<u>B</u>) in various types of tissues by Northern blot hybridization. The poly(A)$^+$ RNA samples were electrophoresed in agarose and transferred to a nitrocellulose filter.   The PCR products of NBL6 and merlin were used to probe the RNA blot.   Mobility of RNA standards (kb) is shown at right.   Lane 1, brain;   lane 2,   heart; lane 3,   lung;   lane 4,   liver ; lane 5, thymus;   lane 6,   spleen; lane 7, kidney.   Note that NBL6 is mainly expressed in brain, while merlin expresses preferably both in brain and thymus.   Both Northern blot   analyses revealed three hybridizing species of 2.5kb, 4.0kb and 5.5kb.
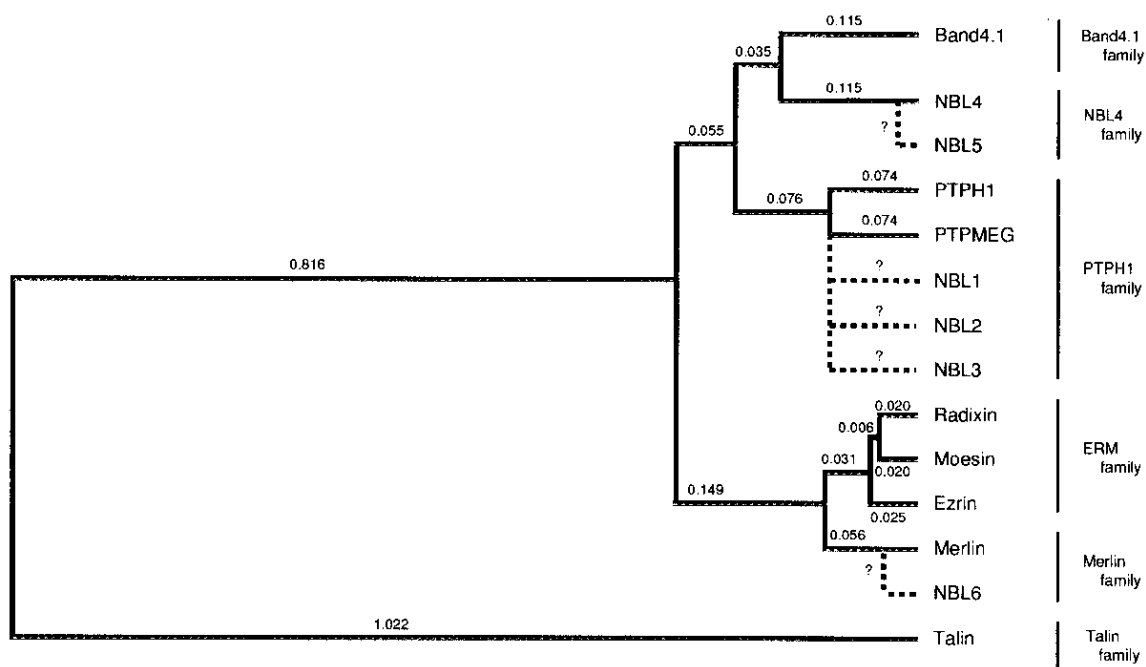
Figure 8. A possible phylogenetic tree of the band 4.1 superfamily. The tree was constructed by the unweighed pair group method (Fitch and Margoliash, 1967; Nei, 1987), using the calculated genetic distances (presented in a numeral) between pairs of members.

Chapter II

Perturbation of Cell Adhesion and Microvilli Formation by Antisense Oligonucleotides to ERM Family Members

Introduction

ERM family consists of three highly-related proteins; ezrin, radixin, and moesin (Sato et al., 1992; Tsukita et al., 1992). Ezrin (also called cytovillin or p81) was first identified as a constituent of microvilli in intestinal epithelial cells (Bretscher, 1983; Pakkanen et al., 1987), and recently revealed to be a good substrate in vivo for tyrosine kinases such as EGF receptors (Bretscher, 1989; Gould et al., 1986; Hunter and Cooper, 1981, 1983). Radixin is a barbed-end capping actin modulating protein which was first purified and characterized from the isolated cell-to-cell adherens junction fraction (Tsukita et al., 1989). Moesin was originally identified as an extracellular heparin-binding protein (Lankes et al., 1988) and was later found to be an intracellular protein (Lankes and Furthmayr, 1991). Since these proteins were very similar to each other, the identity of each protein was not fully established until each cDNA was cloned and sequenced (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991; Sato et al., 1992).

Sequence analyses revealed that these three proteins are highly homologous; in mouse 75, 72, and 80% identity for ezrin/radixin, ezrin/moesin, and radixin/moesin, respectively (Funayama et al., 1991; Sato et al., 1992). Especially, the sequence of their N-terminal half region is highly conserved (~85% identity for any pair). This conserved sequence was also found in the N-terminal region of the band 4.1 superfamily members such as band 4.1 protein, talin, protein-tyrosine-phosphatases (PTPH1 and PTPMEG), indicating that the ERM family is included in the band 4.1 superfamily (Conboy et al., 1986; Rees et al., 1990; Gu et al., 1991; Yang et al., 1991). Detailed analysis with in vitro translation using each cDNA revealed that mouse ezrin, radixin, and moesin exhibit 85, 82, 75kD in SDS-

PAGE, making it possible to compare the expression pattern of these ERM family members in various types of cells and tissues by immunoblotting (Sato et al., 1992). In all types of mouse cultured cells ever examined, ezrin, radixin, and moesin were all coexpressed, while in tissues their expression pattern was distinct from tissue to tissue.

The distribution of ERM family members inside cells has been so far intensively analyzed in various combinations of antibodies and cells (Bretscher, 1983; Pakkanen et al., 1987; Tsukita et al., 1989; Sato et al., 1991, 1992; Lankes et al., 1988). However, the results obtained did not appear to be consistent with each other. This is probably because in many cases: (a) the identity for each member was confused, (b)the antibodies used were not specific for each member, and (c) the masking problem for immunofluorescence microscopy was severe in the case of ERM family members. Taking all localization works on ERM family members into consideration, what we can conclude at present is that at least one ERM family member is concentrated in the plasmalemmal undercoat at cell-to-cell adherens junctions, cell-to-substrate adherens junctions, microvilli, ruffling membranes, and cleavage furrows (Sato et al., 1992). These are specialized sites where actin filaments are densely associated with plasma membranes, leading us to the idea that ERM family members play a crucial role in the association between actin filaments and plasma membranes in general. This idea seems to be consistent with biochemical data. The N-terminal half of band 4.1 protein, which shows a similarity to the N-terminal half of ERM family members, is known to be responsible for its direct binding to an integral membrane protein called glycophorin C in erythrocytes (Anderson and Lovrien, 1984; Aderson and Marchesi, 1985; Leto et al., 1986), suggesting that ERM family members are directly associated with a putative inte-

gral membrane protein. On the other hand, radixin was shown to directly interact with actin filaments at least in vitro (Tsukita et al., 1989), and the C-terminal half of ezrin was reportedly associated with actin filaments in vivo (Algrain et al., 1993).

The significance of the coexpression of more than two ERM family members in single cells is not clear at present. It could be that the ERM family members are sorted out into specific regions. Another possibility is that this coexpression is a redundancy for the sake of safety. In this study, to answer this question, and to directly analyze the physiological functions of each member, we constructed antisense phosphorothioate oligonucleotides (PONs) complementary to the sequence of each ERM family member and applied it to cultured epithelial cells (MTD-1A cells) and thymoma cells (L5178Y). Antisense PONs are now known to be so stable that they can effectively suppress the expression of corresponding proteins (Matsukura et al., 1987; Paria et al., 1992; Ratajczak et al., 1992; Yokozaki et al., 1993; Osen-Sand et al., 1993). When these cells were cultured in the presence of antisense PONs complementary to the ezrin, radixin, or moesin sequence, the expression of the corresponding ERM family member was selectively suppressed. Immunofluorescence microscopy of these antisense-treated cells enabled us to conclude that all the members are colocalized at cell-to-cell adherens junctions, microvilli, and cleavage furrows. We found that these antisense PONs perturbed cell-substrate adhesion, cell-cell adhesion, and microvilli formation. This perturbation was observed most clearly when the ezrin/radixin/moesin antisense PONs mixture was added into culture medium. We believe this study will give us a clue to a better understanding of the significance of the coexpression of more than two ERM family members in single cells and of the physiological functions

of ERM family members.

Materials and Methods

Antibodies

Three distinct antibodies against ERM family members were used; mAb M11 which was raised in rat against recombinant mouse ezrin produced in E.coli (Kasahara et al., manuscript in preparation), pAb I1 which was raised in rabbit against purified rat radixin (Tsukita et al., 1989), and mAb M22 which was raised in rat against recombinant mouse moesin produced in E.coli (Kasahara et al., manuscript in preparation). The mouse anti-ZO-1 mAb (T8-754) was previously reported (Itoh et al., 1991). Anti-mouse E-cadherin pAb was a generous gift from Dr.M.Takeichi (Kyoto University).

Cell Culture

Mouse mammary tumor MTD-1A cell line is a subclone isolated from the original MTD-1 line (Enami et al., 1984; Hirano et al., 1987). Mouse thymoma cell line (L5178Y), mouse myeloma cell line (P3), and mouse leukemia cell line (WEHI231) were generous gifts from Dr.I.Yahara (Tokyo Metropolitan Institute for Medical Sciences), Dr.T.Obinata (Chiba University), and Dr.T.Yamamoto (Tokyo University), respectively. MTD-1A cells were cultured in DMEM supplemented with 10% FCS, and for the other cells RPMI-1640 with 10% FCS were used. To minimize the amount of antisense PONs, the 24 well or 96 well plastic dishes, or 16 well Chamber Slide dishes whose bottom was made of glass (Nunc, Inc. Naperville, IL) were used.

For replating experiments of MTD-1A cells, cells cultured on dishes were treated with 5 mM EDTA in PBS for 10 min followed by incubation with PBS containing 0.25% trypsin for 5 min. After floated cells were washed with culture medium, they were plated on culture dishes or glass cover slips in

culture medium.

For Ca^{++}-switch experiments, MTD-1A cells were cultured in Eagle's minimal essential medium containing 0.05mM Ca^{++} and 10% FCS (low-Ca medium) for 48hr. In the case of the low-Ca medium, FCS was pretreated with chelex to remove Ca^{++} before use. They were then transferred to DMEM supplemented with 10% FCS (normal-Ca medium).

Antisense Phosphorothioate Oligonucleotides (PONs)

First, two distinct positions 1-24 and 301-324 (relative to the translation initiation site) of radixin sequence were chosen for the synthesis of antisense PONs, and we found that antisense PONs complementary to the former position was highly effective. Therefore, we synthesized antisense PONs complementary to position 1-24 of mouse ezrin, radixin, and moesin coding region (see Fig.1). For control experiments, sense PONs corresponding to position 1-24 were synthesized. All effects induced by antisense PONs in this study were totally canceled by the addition of 1.5 vol. of sense PONs. The antisense PONs sequences did not have significant homology with any other sequence in the database.

The antisense and sense PONs were synthesized on an Applied Biosystems 392 synthesizer (Applied Biosystems, Inc. Foster City, CA) in our laboratory or in the laboratory of Takara Shuzo Co. (Kyoto, Japan), purified over Aquapore RP-300 (Applied Biosystems, Inc. Foster City, CA), ethanol precipitated, and taken up in media. At the beginning of the treatment, antisense or sense PONs were added to the culture medium at the concentration of 20 μM, and at every 3-4hr the same amount of PONs were added. At every 4 times of PONs addition, the culture medium was exchanged with the new medium containing 20μM PONs.

Gel Electrophoresis and Immunoblotting

One-dimensional SDS-PAGE (10 %) was based on the method of Laemmli (1970) and the gels were stained with Coomasie Brilliant Blue R-250 or silver staining kit (Wako Pure Chemical Industries, Osaka, Japan).

For immunoblotting, after electrophoresis, proteins were electrophoretically transferred from gels to nitrocellulose sheets, which were then incubated with the first antibody. For antibody detection, a blotting detection kit (Amersham, Corp., Arlington Heights, IL) was used.

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy of cultured cells, cells were cultured on cover glasses or in Chamber Slide dishes whose bottom was made of glass (Nunc, Inc., Naperville, IL), fixed with 1% formaldehyde in PBS for 15 min at room temperature, then treated with 0.2% Triton X-100 in PBS for 10 min. After samples were treated with PBS containing 1% BSA for 10 min, they were then incubated with first antibodies and washed with PBS followed by incubation with second antibodies. As second antibodies, FITC-conjugated goat anti-rat IgG, FITC-conjugated goat anti-rabbit IgG (TAGO, Inc., Burmingame, CA), and/or rhodamine-conjugated goat anti-mouse IgG (CHEMICON INTERNATIONAL, Inc., Temecula, CA) were used. Samples were then washed with PBS and then examined using a fluorescence microscope, a Zeiss Axiophoto photomicroscope (Carl Zeiss, Oberkochen, Germany).

Scanning Electron Microscopy

Thymoma cells were put on the poly-L-lysine coated-cover slips in the RPMI medium for 5 min, and then fixed with 0.2 M cacodylate buffer solution (pH 7.2) containing 2.5% glutaraldehyde and 2% formaldehyde at 4°C over-

night followed by postfixation with the same buffer solution containing 1% OsO_4 at 4℃ for 1hr. Samples were then washed with distilled water, dehydrated in a graded series of ethanol, transferred into isoamyl acetate, and dried in an Eiko critical point drier (Eiko Engineering, Ibaraki, Japan) after substitution with liquid CO_2. Dried samples were coated with gold by means of an Eiko gold sputter coater (Eiko Engineering, Ibaraki, Japan), and examined in a Hitachi S-800 scanning electron microscope (Hitachi Co., Ibaraki, Japan).

Results

Effects of Antisense Phosphorothioate Oligonucleotides on the Expression of ERM Family Members

By preliminary immunohistochemical analyses with cultured MTD-1A cells, we have checked whether or not antisense phosphorothioate oligonucleotides (PONs) complementary to two distinct positions 1-24 and 301-324 (relative to the translation initiation site) of mouse radixin coding region suppress the expression of radixin. We found that, when the antisense PONs complementary to position 1-24 of radixin sequence was added in the culture medium at every 3-4 hours at the concentration of 20 μM, the expression of radixin appeared to be most effectively and continuously suppressed. Therefore, we synthesized antisense PONs complementary to position 1-24 of mouse ezrin, radixin and moesin coding regions (Fig.1), and checked the effects of these antisense PONs on the expression of ERM family members in MTD-1A cells and thymoma cells by immunoblotting (Fig.2).

For immunoblotting, pAb I1 was used, which recognizes all the ERM family members in immunoblotting (Sato et al., 1992). When MTD-1A cells were cultured in the presence of ezrin, radixin, or moesin antisense PONs, the expression of corresponding ERM family member was selectively suppressed to the level undetectable with immunoblotting (Fig.2A, lanes 3-5). Furthermore, for example, the addition of the ezrin/moesin antisense PONs mixture in culture medium resulted in the suppression of both ezrin and moesin expression, but in no effects on the radixin expression (Fig.2A, lane 6). The expression of ERM family members was totally suppressed in the presence of the ezrin/radixin/moesin antisense PONs mixture (Fig.2A, lane 7). The same results were obtained with thymoma cells (Fig.2B), and as far as we

examined any sense PONs did not affect the expression of ERM family members both in MTD-1A cells and thymoma cells.

Distribution of ERM Family Members in Antisense-treated Epithelial Cells

As shown in Fig.2, when cultured MTD-1A cells were treated with ezrin, radixin, or moesin antisense PONs for 96hr, the expression of corresponding ERM family member was completely suppressed. As will be shown later (see Fig.4), the morphology of these antisense-treated cells was not significantly affected. We immunofluorescently stained these ezrin, radixin, or moesin "single-null" MTD-1A cells with three distinct antibodies; mAb M11 which was raised in rat against recombinant mouse ezrin, pAb I1 which was raised in rabbit against purified rat radixin, and mAb M22 which was raised in rat against recombinant mouse moesin. In control sense-treated MTD-1A cells, all these antibodies clearly stained cell-to-cell adherens junctions (AJ) at cell-cell borders and microvilli at the apical surface (Fig.3J-L). Anti-ezrin mAb M11 and anti-radixin pAb I1 stained cell-to-cell AJ stronger than microvilli, whereas anti-moesin mAb M22 showed a stronger staining in microvilli than in cell-to-cell AJ. In dividing cells, the cleavage furrows were intensely stained by all these antibodies (data not shown). As shown in Fig.3A-C, in ezrin "single-null" cells, anti-ezrin mAb M11 gave no signals, while anti-radixin pAb I1 and anti-moesin mAb M22 intensely stained cell-to-cell AJ and microvilli. In contrast, in the case of radixin "single-null" cells, the staining pattern with anti-ezrin mAb M11 and anti-moesin mAb M22 was not affected, while anti-radixin pAb I1 stained no structure (Fig.3D-F). Furthermore, in moesin "single-null" cells no signals were detected with anti-moesin mAb M22, while strong signals at cell-to-cell AJ and microvilli with anti-ezrin mAb M11 and anti-radixin

pAb Il.

In summary, also at the immunofluorescence level, antisense PONs selec-tively suppressed the expression of corresponding ERM family member to the undetectable level without affecting the distribution of the other members. Conversely speaking, this indicates that three antibodies used here, mAb M11, pAb Il, and mAb M22, are specific for ezrin, radixin, moesin, respectively, at the immunofluorescence microscopic level: Also in immuno-blotting, mAb M11 and mAb M22 specifically recognize ezrin and moesin, respectively (data not shown), although, as shown in Fig.2, pAb Il is not specific for radixin in immunoblotting. This strict specificity of anti-bodies at the immunofluorescence level leads us to conclude that ezrin, radixin and moesin are all colocalized at cell-to-cell AJ, microvilli, and cleavage furrows in MTD-1A cells, although there is some bias in their localization: Ezrin/radixin and moesin appeared to be sorted out to some extent to cell-to-cell AJ and microvilli, respectively.

Perturbation of Cell-Substrate Adhesion by Antisense PONs

As shown in Fig.4A-C, neither ezrin, radixin, nor moesin antisense PONs singly induced any morphological changes of MTD-1A cells. Any pair of these antisense PONs also showed no effects on their morphology (data not shown). However, when MTD-1A cells were cultured in the presence of the ezrin/radixin/moesin antisense PONs mixture, the significant change of cell shape was gradually induced (Fig.4E-G): Cells rounded up with very thin protrusions at ~48hr incubation, and began to float off from the substratum at ~60hr incubation. These floated cells were viable judging from the trypane blue staining, and in these cells actin and vinculin were diffusely stained by immunofluorescence microscopy (data not shown). The

ezrin/radixin/moesin sense PONs mixture did by no means induce the morphological changes of MTD-1A cells (Fig.4D).

Next, we examined the effect of antisense PONs on the attachment and spreading after replating cells. For this purpose, MTD-1A cells were cultured for 48hr in the presence of ezrin, radixin or moesin antisense PONs, floated off from the substratum by the use of EDTA and trypsin, then replated on the substratum in the presence of antisense PONs (Fig.5). Cells treated singly with radixin antisense PONs completely lost the ability for the attachment and spreading (Fig.5C), and ezrin antisense-treated cells seemed to partly loose the attachment ability (Fig.5B). In sharp contrast, moesin antisense-treated cells normally attached and spread on the substratum (Fig.5D). The ezrin/radixin/moesin sense PONs mixture did not affect this ability (Fig.5A).

Perturbation of Cell-Cell Adhesion by Antisense PONs

As shown in Fig.4E-G, the ezrin/radixin/moesin antisense PONs mixture appears to affect not only the cell-substrate adhesion but also cell-cell adhesion. Then these antisense-treated cells were doubly stained with anti-radixin pAb I1 and anti-ZO-1 mAb just before the beginning of the cell shape change (Fig.6). It was clearly revealed that the destruction of cell-cell adhesion proceeds or simultaneously associated with that of cell-substrate adhesion. Any one or any pair of three antisense PONs did not affect the cell-cell adhesion (data not shown).

Next, we examined whether or not these antisense PONs singly affect the formation of cell-cell adhesion when cells were transferred from low-Ca to normal-Ca medium. MTD-1A cells were cultured for 2 days in the low-Ca medium containing 0.05 mM Ca^{++} in the presence of the ezrin, radixin, or

moesin antisense PONs, and transferred to the normal-Ca medium containing 2mM Ca^{++} in the presence of antisense PONs. In the presence of ezrin or radixin antisense PONs, the concentration of E-cadherin and ZO-1 at cell-cell contact sites was significantly inhibited (Fig.7A-D), whereas moesin antisense PONs did not affect their concentration (Fig.7E,F). The ezrin/radixin/moesin sense PONs mixture did not affect the formation of cell-cell adhesion (Fig.7G,H).

Effects of Antisense PONs on Microvilli in Thymoma Cells

By preliminary immunofluorescence microscopic analyses, 3-days culture of MTD-1A cells in the presence of the ezrin/radixin/moesin antisense PONs mixture did not appear to induce significant structural changes of micro-villi, where ezrin, radixin and moesin are co-concentrated. Since further culture under this condition resulted in the detachment of MTD-1A cells followed by cell death probably due to their anchorage dependency, it was difficult to pursue the prolonged (>3days) effects of this antisense PONs mixture on the microvilli formation as far as MTD-1A cells were used. Therefore, we used mouse thymoma cells (L5178Y), which bear a large number of microvilli and show no anchorage dependency (Fig.8).

As shown in Fig.9A,B by scanning electron microscopy, when thymoma cells were cultured in the presence of ezrin or radixin antisense PONs, no change was detected in length and number of microvilli even 6 days after the beginning of antisense treatment. Moesin antisense PONs singly appeared to decrease the number and length of microvilli at 6-day culture, but the extent of this effect varied from cell to cell (Fig.9C). In sharp contrast, the addition of the ezrin/radixin/moesin antisense PONs mixture clearly affected the microvilli structures (Fig.9D). At 4 days after the

beginning of the treatment, microvilli began to decrease in number and length (Fig.10A,B). At 6 days, all cells were completely devoid of microvilli (Fig.9D,10C). The trypane blue staining revealed that these cells were viable. With the ezrin/radixin/moesin sense PONs mixture, all cells bore a large number of microvilli (Fig.8). When mouse myeloma cells P3 and mouse leukemia cells WEHI231 were used, the same results were obtained (data not shown).

Discussion

We have examined effects of ezrin, radixin, and moesin antisense PONs on the morphology of cultured cells such as MTD-1A cells and thymoma cells (L5178Y). Ezrin, radixin and moesin (ERM family members) are highly homologous to each other in their amino acid sequences (more than 70% identity). Nevertheless, each antisense PONs selectively suppressed the expression of the corresponding ERM member to the undetectable level. Therefore, this system enabled us to analyze in detail the function of each ERM family member.

Using this system, we produced ezrin, radixin, or moesin "single-null" MTD-1A cells. In these "single-null" cells, the antisense-suppression of one member did not affect the localization of the other two members; they were concentrated at cell-to-cell AJ, microvilli, and cleavage furrows. Interestingly, there appeared to be some bias in the localization between ezrin/radixin and moesin: Ezrin/radixin and moesin showed a tendency to be sorted out to some extent to cell-to-cell AJ and microvilli, respectively. As discussed previously, the detection of ERM family members at cell-to-substrate AJ was difficult probably due to the masking problem (Sato et al., 1992). These findings exclude the possibility that ERM family members are completely sorted out into their specific sites inside cells. Furthermore, the "double-null" cells, ex. ezrin(-)/radixin(-)/moesin(+) cells, were also produced. Even in these cells, the expressing ERM family member is singly concentrated at cell-to-cell AJ, microvilli and cleavage furrows (data not shown). In another word, each member by itself has an ability to concentrate in specific sites. The N-terminal half of ERM family members was highly conserved (~85% identity) and this domain

was thought to be responsible for the specific binding of ERM family members to a putative integral membrane protein (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmyr 1991). If all the ERM family members share the same membrane protein, the redundant colocalization of ERM family members observed here would be easily explained. However, this interpretation may be oversimplified, because, as discussed above, ezrin/radixin and moesin are sorted out to some extent.

The localization of ERM family members suggested that they play crucial roles in the formation and maintenance of cell-cell/cell-substrate adhesion, microvilli, and cleavage furrows by regulating the actin filament-plasma membrane interactions (Sato et al., 1992). This study revealed that the antisense-treatment was very powerful to experimentally evaluate this speculation on the physiological functions of ERM family members. In general, to completely and continuously suppress the expression of structural proteins such as ERM family members, the concentration of antisense PONs in the medium must be maintained at a relatively high level for several days, probably due to their low turnover rate. In this study, we found that antisense PONs should be added into the culture medium at every 3-4hr at the concentration of 20 μM. Under this condition, antisense PONs effectively suppressed the expression of ERM family members and perturbed cell adhesion and microvilli formation.

As to the perturbation of cell adhesion, two points should be discussed. The first point is that ERM family members appear to be involved preferably in the initial step of the formation of cell-substrate/cell-cell adhesion rather than in the maintenance of the preformed adhesion. Any one or any pair of antisense PONs did not destroy the preformed cell-substrate and cell-cell adhesion of MTD-1A cells, whereas the ezrin or radixin "single-

null" cells showed a decreased ability not only to attach and spread on the substratum when replated but also to adhere to each other when transferred from low-Ca to normal-Ca medium. This appears to be consistent with the previous observation that ERM family members are highly concentrated at the so called microspikes of spreading cells which were considered as the primordial cell-to-substrate AJ (Bretscher, 1983; Rinnerthaler et al., 1988; Sato et al., 1992). Of course, the occurrence of at least one ERM family member is required for the maintenance of preformed cell adhesion, because the addition of ezrin/radixin/moesin antisense PONs mixture completely destroyed both preformed cell-substrate and cell-cell adhesion (see Fig.4,6).

The second point is that the physiological role of moesin appears to be slightly different from that of ezrin and radixin. Actually, unlike the ezrin and radixin "single-null" cells, the moesin "single-null" cells normally attached and spread on the substratum when replated, and formed cell-cell adhesion when transferred from low-Ca to normal-Ca medium. However, moesin is somehow involved in cell adhesion, because the ezrin/radixin antisense PONs mixture did not affect the preformed cell adhesion whereas the ezrin/radixin/moesin antisense PONs mixture destroyed it completely. Therefore, it is likely that between ezrin/radixin and moesin there is some synergetic interaction in the regulation of cell adhesion. In this connection, it should be mentioned that among ERM family members, only moesin lacks the polyproline stretch at their C-terminal region (Lankes and Furthmyr 1991; Sato et al., 1992), and that, as mentioned above, the localization of moesin is slightly different from that of ezrin/radixin.

As compared to the cell adhesion, the microvillus appears to be more resistant to the antisense-treatment. At 3-4day-culture in the presence

of the ezrin/radixin/moesin antisense PONs mixture microvilli gradually decreased in number and length, and only at 6day-culture they completely disappeared. As compare to the perturbation of cell-cell and cell-sub-strate adhesion, the complete suppression of the expression level of ERM family members appears to be required for the disappearance of microvilli. This microvilli disappearance was most clearly observed in thymoma cells, but similar results were obtained by the use of WEHI231 leukemia cells and P3 myeloma cells (data not shown).

Considering that ERM family members are concentrated at cleavage fur-rows, the question has naturally arisen whether or not antisense PONs to ERM family members affect the cytokinesis. If they affect the cytokine-sis, it can be easily expected that the cells with more than two nuclei increase in number. However, as far as we examined under any conditions with antisense PONs, the two-nuclei cells have not been increased in num-ber. This may be explained in two ways: The first explanation is that ERM family members are not involved in cytokinesis. The second one is that antisense PONs affect not only M-phase but also other phases of the cell cycle under the condition such as the 6day-culture in the presence of the ezrin/radixin/moesin antisense PONs mixture. Actually, the 6day-cultured thymoma cells lacking microvilli appeared to loose the ability to increase in number. Therefore, the latter explanation may be the case, indicating that using antisense PONs we appeared to be unable to experimentally answer the above question. However, taking all findings in this study together with the recent observation that the microvilli rich in ERM family members are highly concentrated at cleavage furrows in dividing cells (Yonemura et al., 1993), it appears to be reasonable to speculate that ERM family members are directly involved in the formation of cleavage furrows.

Another approach will be required to prove this speculation.

Finally, we should discuss the relationship between ERM family members and the regulation of cell growth. Recently, a tumor suppressor responsible for neurofibromatosis 2 was identified and this protein named merlin was found to be similar to ERM family members (~49% identity for full length; ~62% identity for N-terminal half)(Trofatter et al., 1993; Rouleau et al., 1993). Therefore, it is likely that the function of merlin is somehow related to that of ERM family members. Further studies on the function of merlin will lead us to a better understanding of how ERM family members are involved in the regulation of cell growth.

Our present results favored the idea that the coexpression of ERM family members, especially of ezrin and radixin, in single cells is a redundancy for the sake of safety. This type of redundancy generally interferes with the analysis of protein functions in vivo, but as shown in this study the antisense PONs are very convenient tools to escape the interference by redundancy. By this method, in spite of redundancy, we can conclude that ERM family members are directly involved in the cell adhesion and microvilli formation. Further detailed analyses of the structure and functions of the ERM family members "single-null", "double-null" and "triple-null" cells will lead us to a better understanding of how the ERM family members-mediated actin filament/plasma membrane interactions are involved in cell adhesion, cell proliferation, and signal transduction.

References

Algrain,M., O.Turunen, A.Vaheri, D.Louvard, and M.Arpin. 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. J.Cell Biol. 120:129-140.

Anderson,R.A. and R.E.Lovrien. 1984. Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. Nature 307:655-658.

Anderson,R.A. and V.T.Marchesi. 1985. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. Nature 318:295-298.

Bretscher,A. 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. J.Cell Biol. 97:425-432.

Bretscher,A. 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. J.Cell Biol. 108:921-930.

Conboy,J., Y.W.Kan, S.B.Shohet, and N.Mohandas. 1986. Molecular cloning of protein 4.1, a major structural element of the human erythrocyte membrane skeleton. Proc.Natl.Acad.Sci.USA 83:9512-9516.

Doolitle,R.F. 1984. In Putnam,F.W. (ed.), The Plasma Proteins. Academic Press., Orlando,FL, Vol.IV, pp.317-360.

Enami,J., S.Enami, and M.Koga. 1984. Isolation of an insulin-responsive preadipose cell line and a mammary tumor-producing, dome-forming epithelial cell line from a mouse mammary tumor. Dev.Growth Differ. 26:223-234.

Funayama,N., A.Nagafuchi, N.Sato, Sa.Tsukita, and Sh.Tsukita. 1991. Radixin is a novel member of the band 4.1 family. J.Cell Biol. 115:1039-1048.

Gould,K.L., A.Bretcher, F.S.Esch, and T.Hunter. 1989. cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. EMBO J. 8:4133-4142.

Gould,K.L., J.A.Cooper, A.Bretscher and T.Hunter. 1986. The protein-tyrosine kinase substrate, p81, is homologous to a chicken microvillar core protein. J.Cell Biol. 102:660-669.

Gu,M., J.D.York, I.Warshawsky, and P.W.Majerus. 1991. Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. Proc.Natl.Acad.Sci.USA 88:5867-5871.

Hirano,S., A.Nose, K.Hatta, A.Kawakami, and M.Takeichi. 1987. Calcium-dependent cell-cell adhesion molecules (cadherins): Subclass specificities and possible involvement of actin bundles. J.Cell Biol. 105:2501-2510.

Hunter,T. and J.A.Cooper. 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell 24:741-752.

Hunter,T. and J.A.Cooper. 1983. Role of tyrosine phosphorylation in malignant transformation by viruses and in cellular growth control. Prog.Nucleic Acid Res.Mol.Biol. 29:221-233.

Lankes,W., and H.Farthmayr. 1991. Moesin: A member of the protein 4.1-talin-ezrin family of proteins. Proc.Natl.Acad.Sci.USA 88:8297-8301.

Lankes,W., A.Griesmacher, J.Grunwald, R.Schwartz-Albiez, and R.Keller. 1988. A heparin-binding protein involved in inhibition of smooth-muscle cell proliferation. Biochem.J. 251:831-842.

Laemmli,U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Leto,T.L., I.Correas, T.Tobe, R.A.Anderson, and W.C.Horne. 1986. In Membrane Skeleton and Cytoskeletal Membrane Associations. V.Bennet, C.M.Cohen, S.E.Lux, and J.Palek, eds. Alan R.Liss, New York. 201-209,

Matsukura,M., K.Shinozuka, G.Zon, H.Mitsuya, M.Reitz, J.S.Cohen, and S.Broder. 1987. Phosphorothioate analogs of oligodeoxynucleotides: Inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc.Natl.Acad.Sci.USA 84:7706-7710.

Osen-Sand,A., M.Catsicas, J.K.Staple, K.A.Jones, G.Ayala, J.Knowles, G.Grenningloh, and S.Catsicas. 1993. Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. Nature 364:445-448.

Pakkanen,R., K.Hedman, O.Turunen, T.Wahlstrom, and Vaheri,A. 1987. Microvillus-specific Mr 75,000 plasma membrane protein of human choriocarcinoma cells. J.Histochem.Cytochem. 135:809-816.

Paira,B.C., S.K.Dey, and G.K.Andrews. 1992. Antisense c-myc effects on preimplantation mouse embryo development. Proc.Natl.Acad.Sci.USA 89:10051-10055.

Ratajczak,M.Z., J.A.Kant, S.M.Luger, N.Hijiya, J.Zhang, G.Zon, and A.M.Gewirtz. 1992. In vivo treatment of human leukemia in a scid mouse with c-myb antisense oligodeoxynucleotides.

Rees,D.J.G., S.E.Ades, S.J.Singer, and R.O.Hynes. 1990. Sequence and domain structure of talin. Nature 347:685-689.

Rinnerthaler,G., B.Geiger, J.V.Small. 1988. Contact formation during fibroblast locomotion: Involvement of membrane ruffles and microtubules. J.Cell Biol. 106:747-760.

Rouleau,G.A., P.Merel, M.Lutchman, M.Sanson, J.Zucman, C.Marineau, K.Hoang-Xuan, S.Demczuk, C.Desmaze, B.Plougastel, S.M.Pulst, G.Lenoir, E.Bijl-sma, R.Fashold, J.Dumanski, P.de Jong, D.Parry, R.Eldrige, A.Aurias, O.Delattre, and G.Thomas. 1993. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 363:515-521.

Sato,N., N.Funayama, A.Nagafuchi, S.Yonemura, Sa.Tsukita, and Sh.Tsukita. 1992. A gene family consisting of ezrin, radixin, and moesin. Its specific localization at actin filament/plasma membrane association sites. J.Cell Sci. 103:131-143.

Sato,N., S.Yomemura, T.Obinata, Sa.Tsukita, and Sh.Tsukita. 1991. Radixin, a barbed end-capping actub-modulating protein, its concentrated at the cleavage furrow during cytokinesis. J.Cell Biol. 113:321-330.

Trofatter,J.A., M.M.MacCollin, J.L.Rutter, J.R.Murrell, M.P.Duyao, D.M.Parry, R.Eldridge, N.Kley, A.G.Menon, K.Pulaski, V.H.Haase, C.M.Ambrose, D.Munroe, C.Bove, J.L.Haines, R.L.Martuza, M.E.MacDonald, B.R.Seizinger, M.P.Short, A.J.Buckler, and J.F.Gusella. 1933. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 72:791-800.

Tsukita,Sa., Y.Hieda and Sh.Tsukita. 1989a. A new 82 kD-barbed end capping protein localized in the cell-to-cell adherens junction: Purification and characterization. J.Cell Biol. 108:2369-2382.

Tsukita,Sh., Sa.Tsukita, A.Nagafuchi, and S.Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell-to-cell adherens junctions. Curr.Opin.Cell Biol. 4:834-839.

Turunen,O., R.Winqvist, R.Pakkanen, K.H.Grzeschik, T.Wahlstrom, and A.Vaheri. 1989. Cytovillin, a microvillar Mr 75,000 protein. cDNA sequence, pro- karyotic expression, and chromosomal localization. J.Biol.Chem. 264:16727-16732.

Yang,Q. and N.K.Tonks. 1991. Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associat- ed proteins band 4.1, ezrin, and talin. Proc.Natl.Acad.Sci.USA 88:5949-5953.

Yokozaki,H., A.Budillon, G.Tortora, S.Meissner, S.L.Beaucage, K.Miki, and Y.S.Cho-Chung. 1993. An antisense oligodeoxynucleotide that depletes RI alpha subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. Cancer Res. 15:868-872.

Yonemura,S., A.Nagafuchi, N.Sato, and Sh.Tsukita. 1993. Concentration of an integral membrane protein CD43 (Leukosialin, Sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. J.Cell Biol. 120:437-449.

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                    +1                              +24
        5'            ▶                                                3'
Ezrin   · · · · ACCAGCCAAG ATGCCCAAGCCAATCAACGTCCGG GTGACCACCATGGAT · · · ·

Radixin  · · · · GAAAAAGAAA ATGCCGAAGCCAATCAATGTAAGA GTAACTACAATGGAC · · · · ·

Moesin   · · · · TGCCGCCACC ATGCCGAAGACGATCAGTGTGCGT GTCACCACCATGGAT · · · ·
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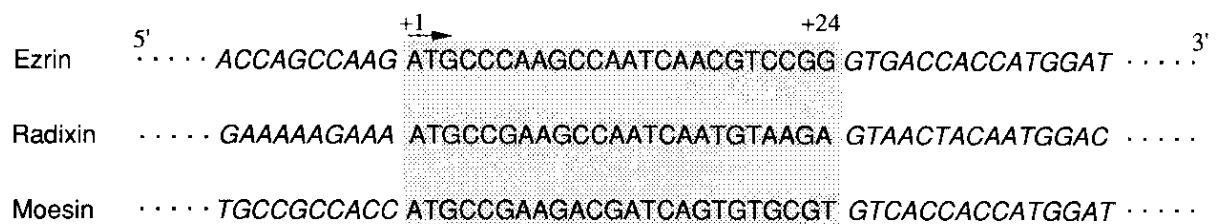
Figure 1. Nucleotide sequences (-10~39) of mouse ezrin, radixin, and moesin (Funayama et al., 1991; Sato et al., 1992). Antisense phosphorothioate oligonucleotides (PONs) complementary to position 1-24 (relative to the translation initiation sites) of ezrin, radixin and moesin were synthesized (shadowed). For control experiments, sense PONs of this region were also synthesized.
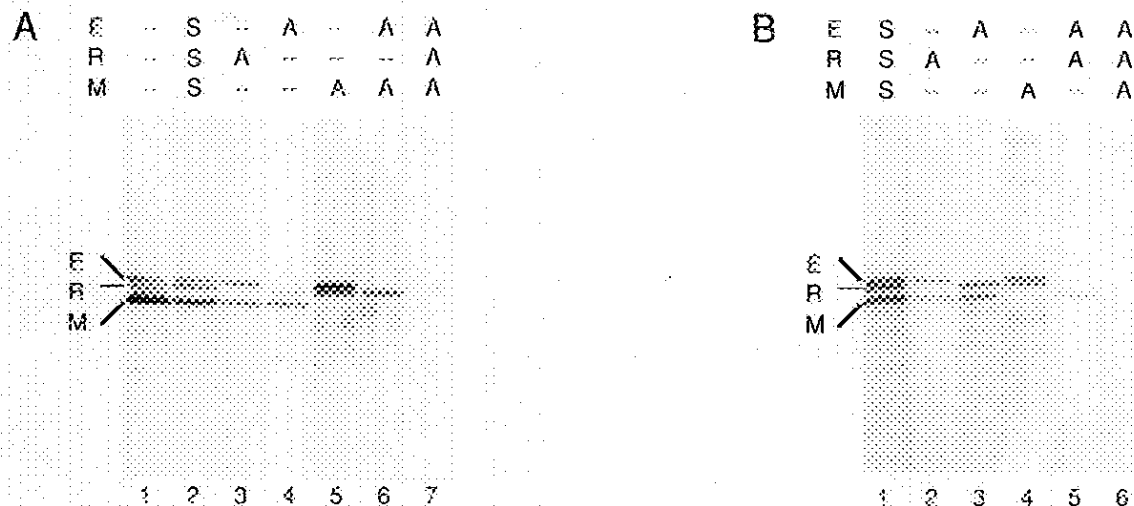
Figure 2. Effects of antisense PONs on the expression of ERM family members in MTD-1A cells (A) and thymoma cells (B). MTD-1A cells and thymoma cells were cultured for 96hr in the presence of various combinations of ezrin, radixin, and moesin antisense/sense PONs in a 24 well dish, separated by SDS-PAGE, and immunoblotted with pAb I1 which can recognize all members of ERM family by immunoblot (E, ezrin of 85kD; R, radixin of 82kD; M, moesin of 75kD). The combination of antisense (A) or sense (S) PONs is shown above each lane. Note that each antisense but not sense PONs selectively suppress the expression of the corresponding ERM family member to the undetectable level. In this system, ERM family member "single-null" (lanes 3-5 in A; lanes 2-4 in B), "double-null" (lane 6 in A; lane 5 in B) and "triple-null" (lane 7 in A; lane 6 in B) cells can be produced.

Figure 3. Distribution of ERM family members in antisense-treated MTD-1A cells. MTD-1A cells were cultured for 96hr in the presence of ezrin (A-C), radixin (D-F) or moesin (G-I) antisense PONs, or in the presence of the ezrin/radixin/moesin sense PONs mixture (J-L), and then immunofluorescently stained with anti-ezrin mAb M11 (A,D,G,J), anti-radixin pAb I1 (B,E,H,K), or anti-moesin mAb M22 (C,F,I,L). In sense-treated cells (J-L), all ERM family members are localized together at cell-to-cell AJ (arrows) and microvilli on apical surfaces (arrowheads). Ezrin/radixin and moesin have a tendency to be to some extent sorted out into cell-to-cell AJ and micro-villi, respectively. In ezrin/radixin (A,B,D,E,G,H,J,K) and moesin (C,F,I,L) staining, the focus plane is fixed at the level of cell-to-cell AJ and of the apical surface, respectively. Even in ezrin/radixin stain-ing, when the apical surface is focused, clear signals are detected from microvilli (ex. arrowheads in D). Note that among antisense-treated cells, only in A,E, and I immunofluorescence signals are undetectable, and that in B-D and F-H no significant changes in the distribution of ERM family members are observed. Staining of nuclei is non-specific, since this staining is not affected by any antisense treatment. Bar, 20μm.
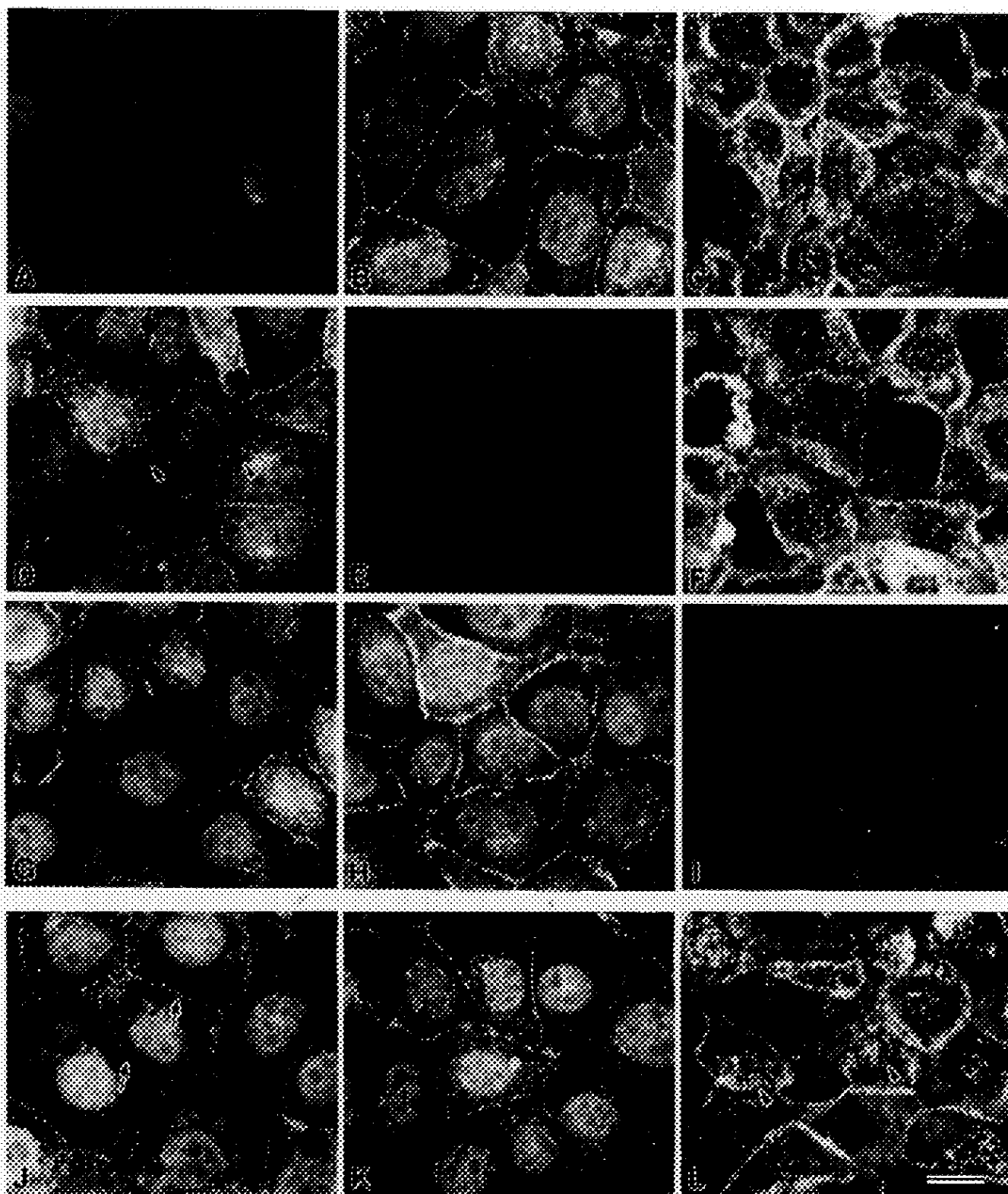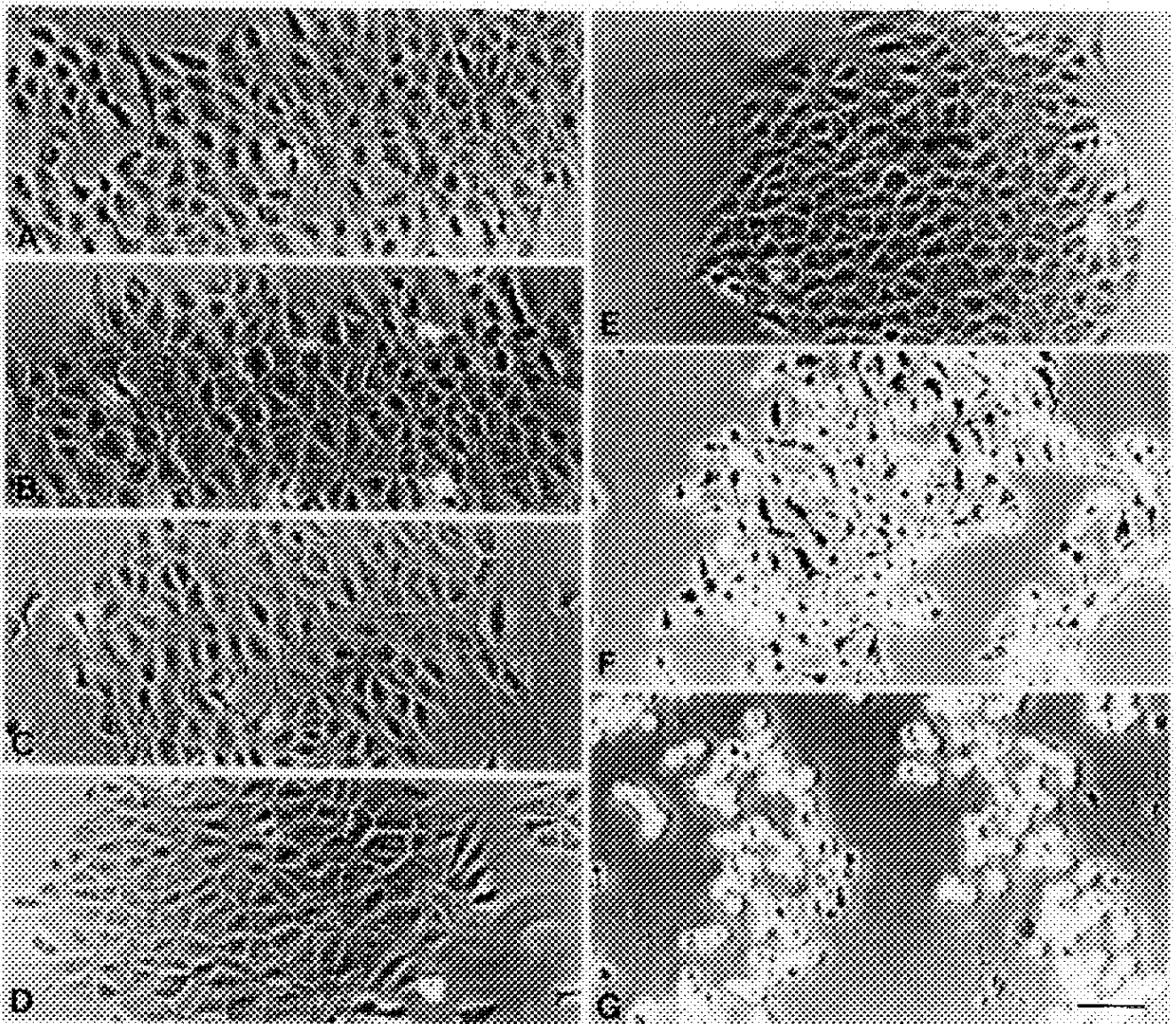
Figure 4. Effects of ERM family member antisense PONs on the cell-substrate adhesion of MTD-1A cells. When MTD-1A cells are cultured for 96hr in the presence of ezrin (A), radixin (B), or moesin (C) antisense PONs, or in the presence of the ezrin/radixin/moesin sense PONs mixture (D), no effects on the cell shape are detectable. In sharp contrast, the ezrin/radixin/moesin antisense PONs mixture induces significant morphological changes: At 24hr-culture (E) no structural changes are observed, whereas at 48hr-culture (F) cells round up with very thin protrusions (arrows) followed by the complete detachment of cells from substratum at 60hr-culture (G). Bar, 100 μm.
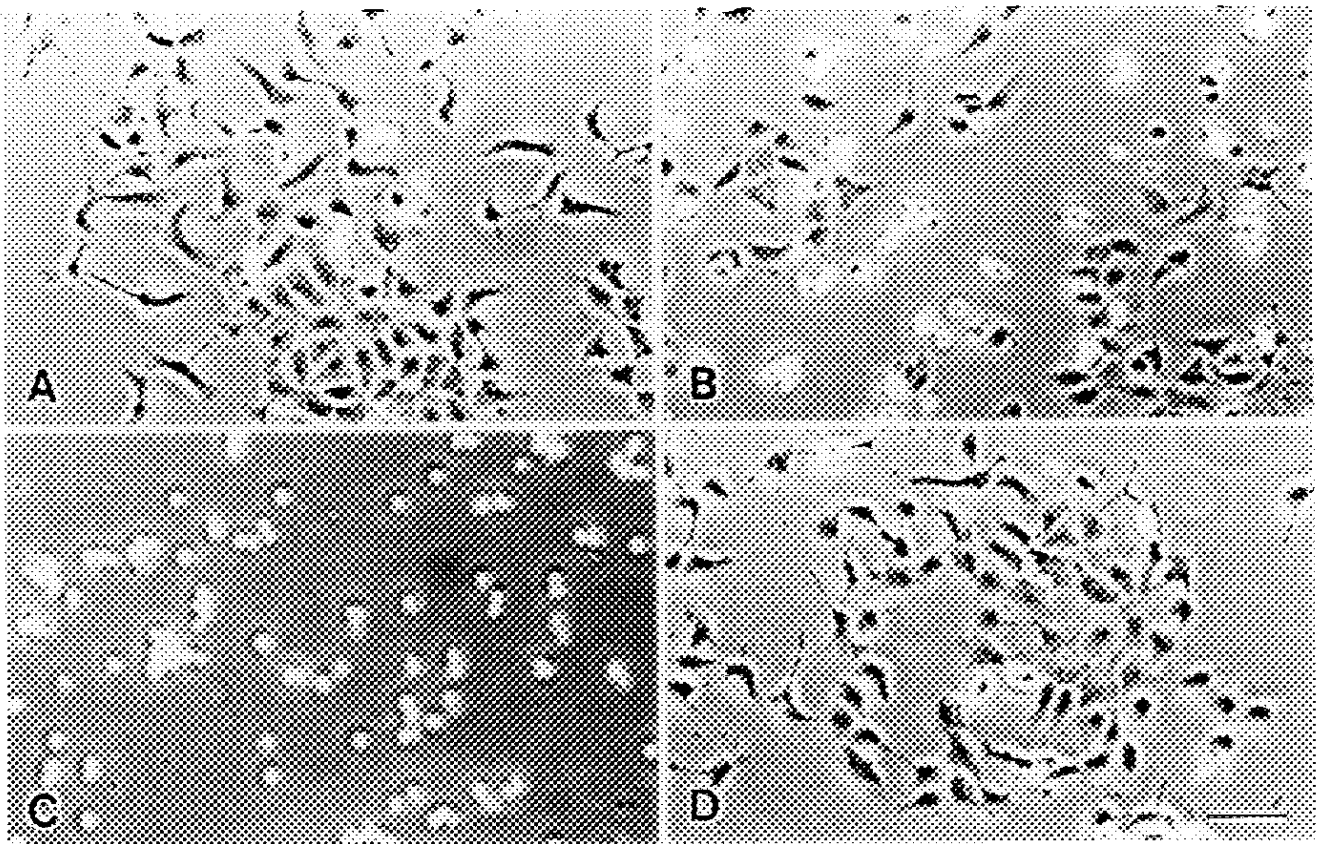
Figure 5. Effects of ERM family member antisense PONs on the adhesion and spreading on substratum after replating MTD-1A cells. MTD-1A cells were cultured for 48hr in the presence of the ezrin/radixin/moesin sense PONs mixture (A) or in the presence of ezrin (B), radixin (C) or moesin (D) antisense PONs, detached from the substratum by the use of EDTA and trypsin, and then replated and cultured on plastic dishes for 15 hr in the presence of same PONs. Moesin antisense-treated cells (D) can normally adhere to and spread on the dish just like sense-treated cells (A), while the ability for adhesion and spreading is suppressed partly in ezrin antisense-treated cells (B) and completely in radixin antisense-treated cells (C). Bar, 100μm.
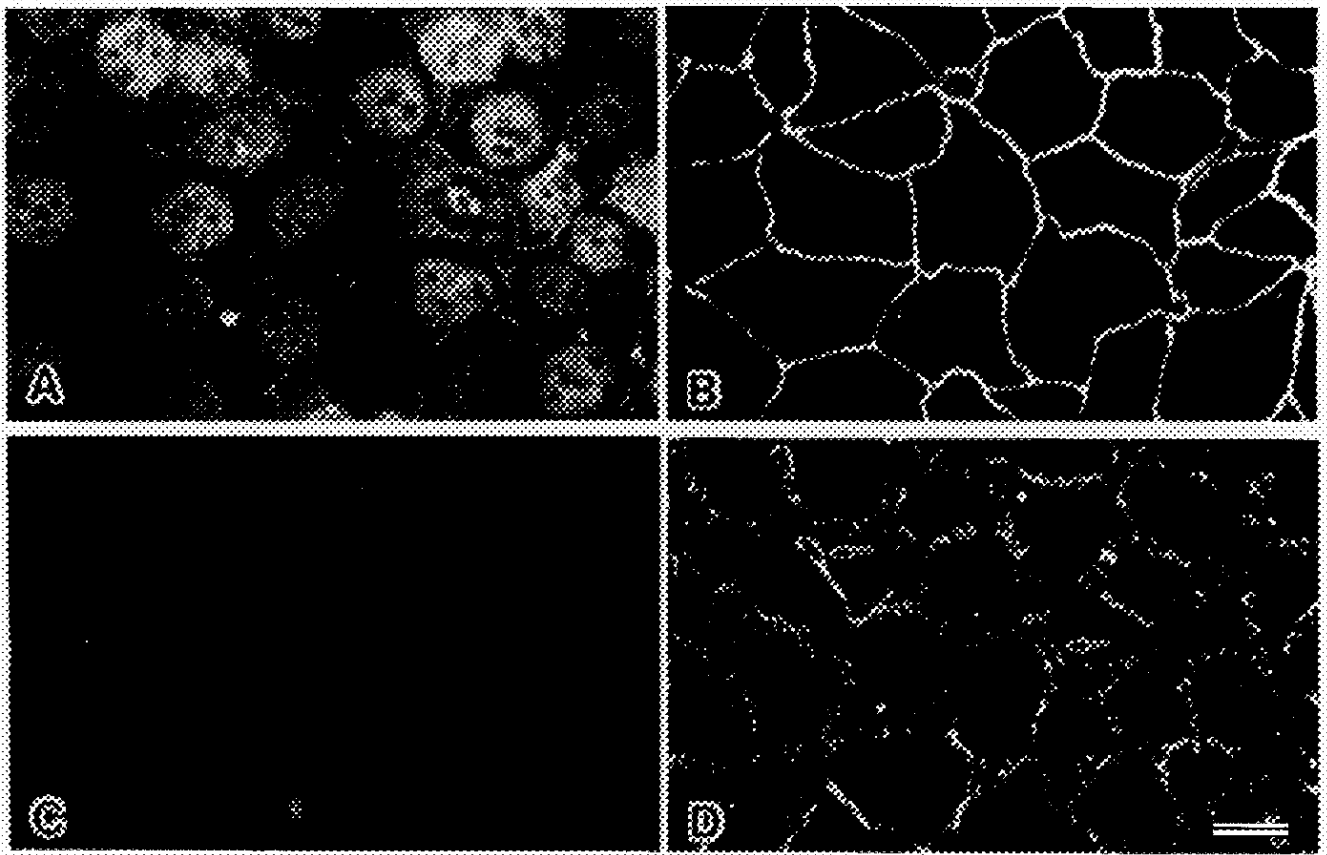
Figure 6. Effects of the ezrin/radixin/moesin antisense PONs mixture on the cell-cell adhesion in MTD-1A cells. As shown in Fig.4E-G, the ezrin/radixin/moesin antisense PONs mixture appears to affect not only the cell-substrate but also the cell-cell adhesion. At 30hr-culture in the presence of the ezrin/radixin/moesin sense PONs mixture (A,B) or the ezrin/radixin/moesin antisense PONs mixture (C,D), cells were doubly stained with anti-radixin pAb I1 (A,C) and anti-ZO-1 mAb (B,D). Note that the suppression of the expression of radixin (and also ezrin and moesin) (C) is accompanied by the destruction of cell-cell adhesion (D). The ZO-1 staining becomes discontinuous followed by its splitting (arrows). Bar, 20 μ m.

Figure 7. Effects of ERM family member antisense PONs on the formation of cell-cell adhesion in MTD-1A cells. In the low-Ca medium containing 0.05 mM Ca^{2+}, MTD-1A cells were cultured for 48hr in the presence of ezrin (A,B), radixin (C,D), or moesin (E,F) antisense PONs, or in the presence of the ezrin/radixin/moesin sense PONs mixture (G,H), and then transferred to the normal-Ca medium containing 2mM Ca^{2+} in the presence of same PONs. At 12 hr after the Ca-switch, cells were stained doubly with anti-E-cadherin pAb (A,C,E,G) and anti-ZO-1 mAb (B,D,F,H). The concentration of E-cadherin and ZO-1 at cell-cell adhesion sites are clearly observed in moesin antisense-treated (E,F) and in sense-treated cells (G,H), whereas hardly detected in ezrin (A,B) and radixin (C,D) antisense-treated cells. Bar, 20 μ m.
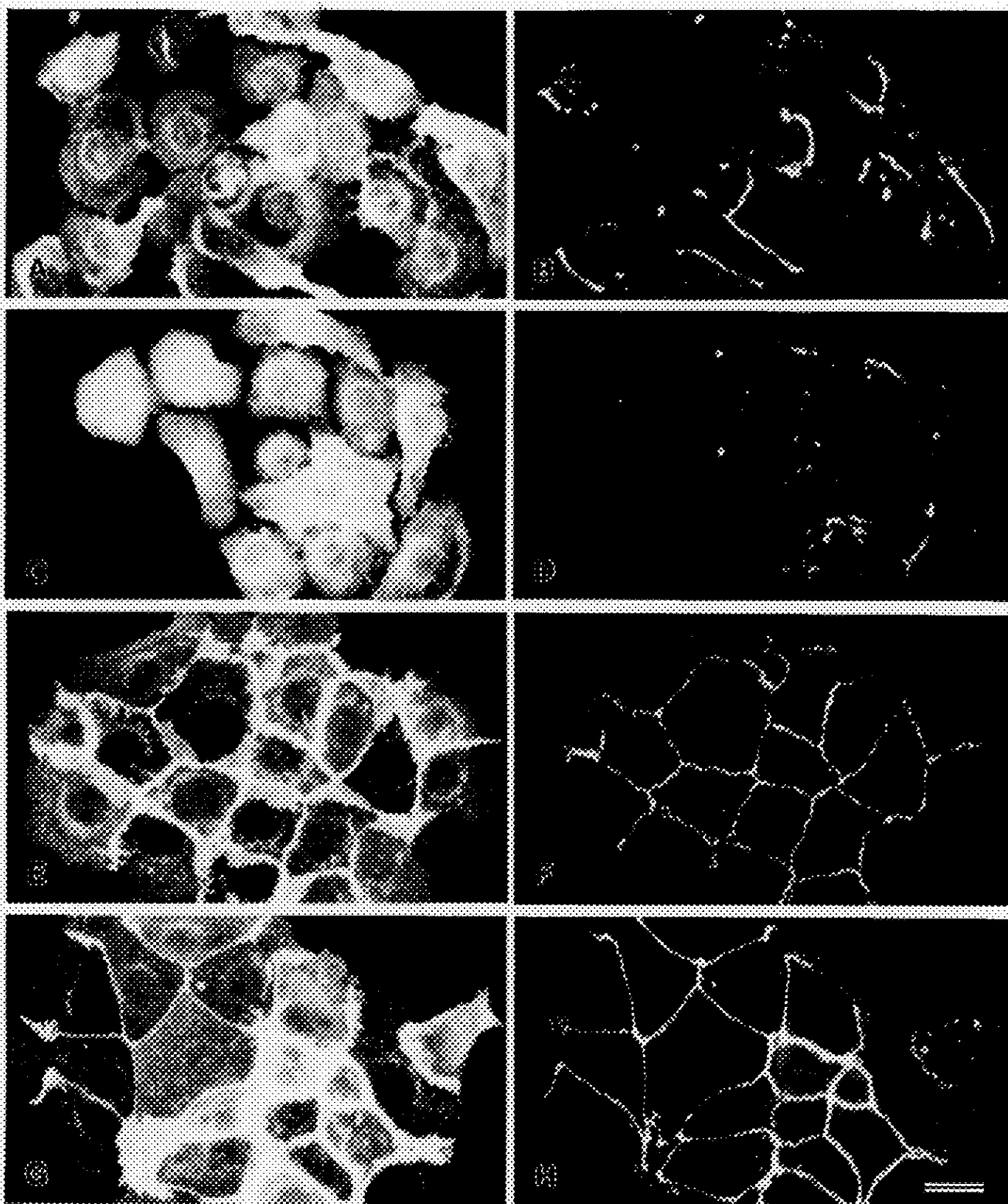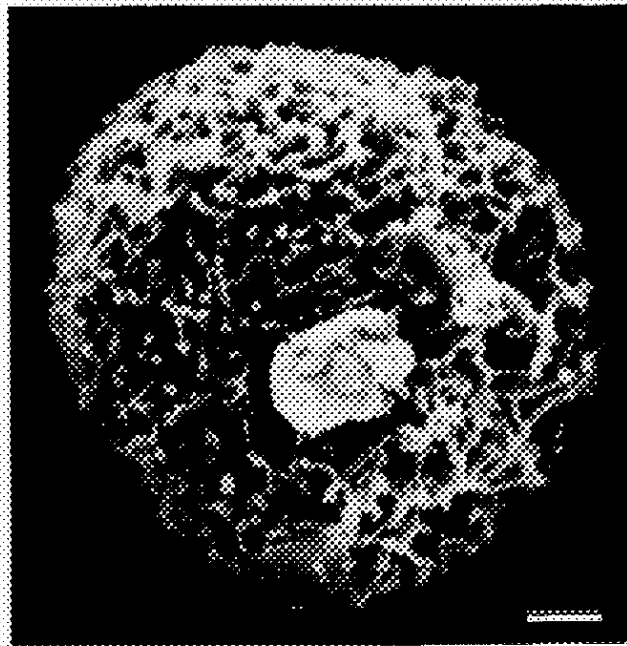
Figure 8. Scanning electron microscopy of thymoma cells treated with the ezrin/radixin/moesin sense mixture for 6 days. Cells are characterized by a large number of microvilli on their cell surface (arrows). Bar, 1μm.
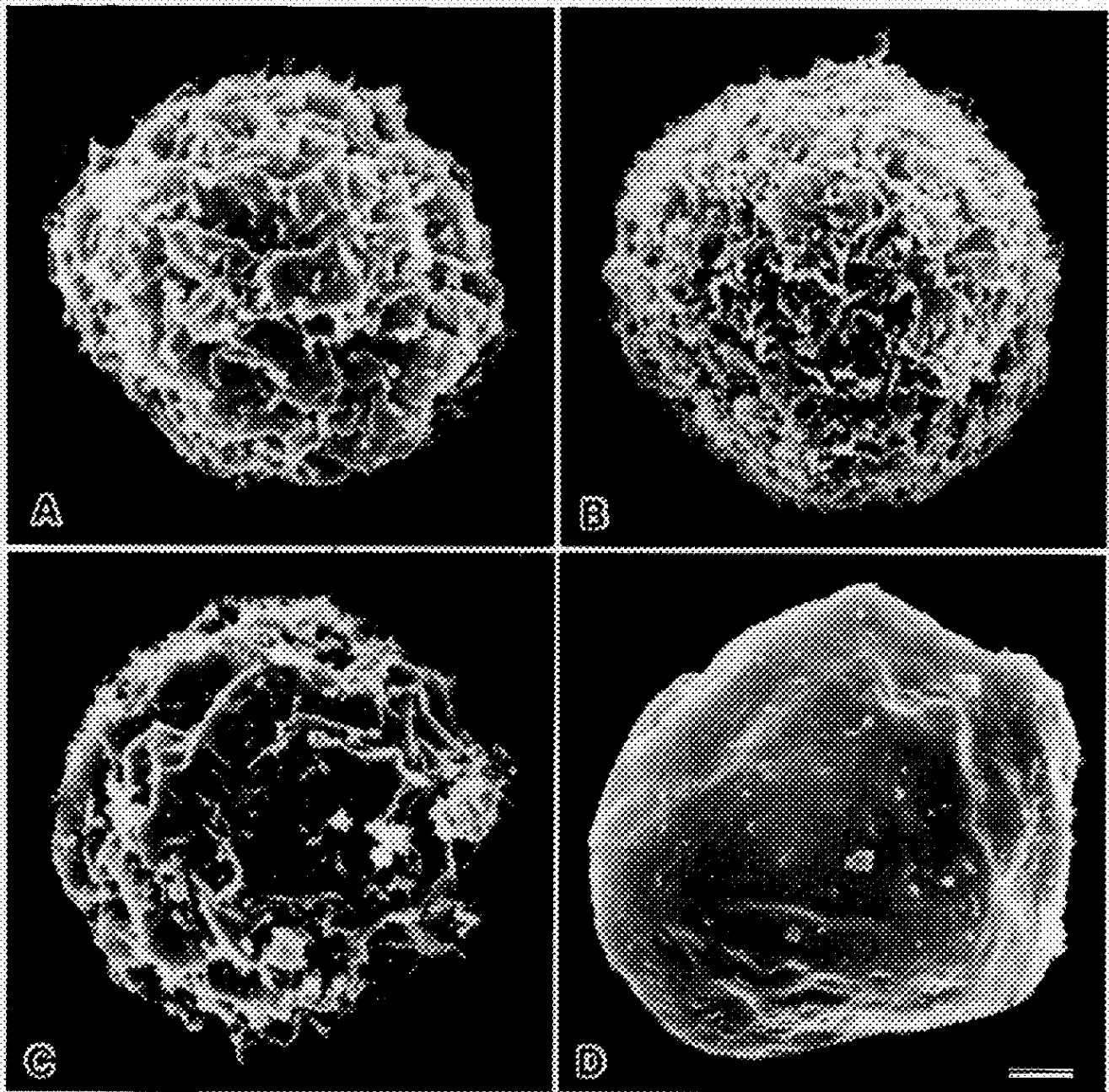
Figure 9. Effects of ERM family member antisense PONs on the microvilli structure of thymoma cells. Thymoma cells were cultured for 6 days in the presence of ezrin (A), radixin (B), or moesin (C) antisense PONs, or in the presence of the ezrin/radixin/moesin antisense PONs mixture (D). Micro-villi are not affected by ezrin (A) and radixin (B) antisense PONs, whereas moesin antisense PONs partly affects them (C). In the presence of the mixture of antisense PONs, microvilli completely disappeared, leaving the smooth cell surface (D). Bar, 1 μm.

Figure 10.   Time   course   of the disappearance of   microvilli   of   thymoma

cells induced by the ezrin/radixin/moesin antisense PONs mixture.   Although

at   the   beginning of the treatment no structural changes are   detected   in

microvilli (A), microvilli begins to decrease in number and length at 4-day

culture   (B).   At 6-day culture, all cells are completely devoid of   micro-

villi (C).   Bar, 2μm.

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