NAG-2, a Novel Transmembrane-4 Superfamily (TM4SF) Protein That Complexes with Integrins and Other TM4SF Proteins*

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Transmembrane-4 superfamily (TM4SF) protein complexes form complexes with integrins and other cell-surface proteins. To further characterize the major proteins present in a typical TM4SF protein complex, we raised monoclonal antibodies against proteins co-immunoprecipitated with CD81 from MDA-MB-435 breast cancer cells. Only two types of cell-surface proteins were recognized by our 35 selected antibodies. These included an integrin (α6β1) and three different TM4SF proteins (CD9, CD63, and NAG-2). The protein NAG-2 (novel antigen-2) is a previously unknown 30-kDa cell-surface protein. Using an expression cloning protocol, cDNA encoding NAG-2 was isolated. When aligned with other TM4SF proteins, the deduced amino acid sequence of NAG-2 showed most identity (34%) to CD53. Flow cytometry, Northern blotting, and immunohistochemistry showed that NAG-2 is widely present in multiple tissues and cell types but is absent from brain, lymphoid cells, and platelets. Within various tissues, strongest staining was seen on fibroblasts, endothelial cells, follicular dendritic cells, and mesothelial cells. In nonstringent detergent, NAG-2 protein was co-immunoprecipitated with other TM4SF members (CD9 and CD81) and integrins (α6β1, and α6β3). Also, two-color immunofluorescence showed that NAG-2 was co-localized with CD81 on the surface of spread HT1080 cells. These results confirm the presence of NAG-2 in specific TM4SF-TM4SF and TM4SF-integrin complexes.

The transmembrane-4 superfamily (TM4SF)1 comprises a group of at least 19 cell-surface proteins (including CD9, CD37, CD53, CD63, CD81, and CD82) each presumed to have four transmembrane domains (1). Although the precise biological functions of TM4SF proteins remain elusive, multiple experiments using anti-TM4SF mAbs have implicated TM4SF proteins in cell proliferation, activation, adhesion, and motility (1, 2). In addition, several TM4SF members may regulate signaling events involving tyrosine phosphorylation (3, 4) and intracellular calcium (5). In the plasma membrane, TM4SF proteins interact with each other (2, 6–10) and form various signaling complexes with other cell-surface proteins including CD4, CD8 (8), CD19 (11), CD20 (12), CD21 (11), MHC class I (12), MHC class II (9, 10, 12), the neural protein L1 (13), and a discrete subset of integrins (6, 7, 13–19). It is hypothesized that within these complexes, a TM4SF protein may function as a new class of membrane adapter that either nucleates assembly and/or regulates signaling activities (20).

Our recent data have indicated that integrin-TM4SF protein complexes contain additional cell-surface components not yet identified. To characterize these proteins we utilized a systematic approach for generating and selecting mAbs against purified integrin-TM4SF complexes. A panel of mAbs was developed against integrin-TM4SF protein complexes that were purified using anti-CD81 mAb-coated beads. The CD81 protein was chosen for immunization because of its widespread distribution and because it is readily identified as a 22-kDa protein following cell-surface labeling. Notably, we found that all selected antibodies recognize either integrins or TM4SF proteins. Moreover, we have identified and characterized a novel TM4SF protein called NAG-2.

MATERIALS AND METHODS

Cell Lines—Human B-cell lines, Raji, JY, and Ramos; T-cell lines, Jurkat and Molt-4; and a promyelocytic cell line, K562 were cultured in RPMI 1640 medium. Chinese hamster ovary (CHO) cell lines were maintained in α-minimum Eagle’s medium, and the human breast carcinoma cell line, MDA-MB-435, the fibrosarcoma cell line HT1080, and all other cell lines were maintained in Dulbecco’s modified Eagle’s medium. All cell lines were supplemented with 10% fetal calf serum. A CHO line (CHO/P) stably transfected with the polyoma large T antigen was used for expression cloning (21). K562α6β2 and α6β3 integrin transfectants (16) as well as an HT1080-CD9 (6) transfectant were previously described. To make CHO-CD63 cells, CHO cells were electroporated with human CD63 cDNA (22) in pCDM8 vector. The CHO-CD63 cells were used for mAb screening 2 days after electroporation.

Antibodies—The anti-TM4SF mAbs used were C9-BB, anti-CD9 (6); SH1 (16) and RUU-SP 2.28 (23), anti-CD63; and M38, anti-CD81 (24). Anti-integrin mAbs were used as 2A2E10, anti-αβ (25); A3-XS, anti-αβ (26); A5-Puj2, anti-αβ (27); A6-ELE, anti-αβ (28); and TS2/16, anti-β (29). Other antibodies were 8E11, anti-CD109 (6); W6/32, anti-MHC class I (30); and P3, negative control antibody (31).

Immunoprecipitation—MDA-MB-435 cells were surface-labeled with NHS-LC-biotin (Pierce) or Na125I according to established protocols and lysed in immunoprecipitation buffer (1% Brij 96 or 1% n-octyl glucoside, 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM MgCl2; 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 1 h at 4 °C. Insoluble materials were pelleted at 12,000 rpm for 10 min, and
the cell lysates were precedure by incubation with pansorbin (Calbiochem) for 30 min at 4 °C. Immune complexes were collected on Sepharose 4B beads (Pharmacia, Uppsala, Sweden) that were pre-bound with mAb, followed by four washes with the immunoprecipitation buffer. For immunoprecipitation under stringent conditions, the Brj 96-immuno-
precipitated cell lysates were subsequently subfractionated three more times, until a single clone confer-
fected with the NAG-2 cDNA was identified. Further subfractionation was performed as described ear-
lier (6). Briefly, protein complexes were immunopurified using anti-
NAG-2 mAb-conjugated Sepharose 4B from nonstringent (without 0.2% SDS) Brj 96 lysates of surface-biotinylated MDA-MB-435 cells. After five washes, the protein complexes were dissociated for 30 min at 4 °C with Brj 96 buffer containing 0.2% SDS. The eluates were subse-
sequently reproprecipitated with anti-TM4SF, anti-integrin mAbs, or control mAbs directly coupled to Sepharose 4B. Reciprocal re-immunoprecipi-
tation experiments (in which NAG-2 was reproprecipitated) were carried out similarly, except that MDA-MB-435 cells were labeled with [3H]Th.

Antibody Production—CD81-containing protein complexes were pu-
riified on mAb M38-coupled Sepharose 4B beads from Brj 96 cellular lysates prepared from MDA-MB-435. After washing, immunocom-
plex-associated Sepharose beads were used for immunization of a RBF/
DnJ mouse. After three injections (each time with complexes derived from 1 to 2 × 10⁷ cells), mouse serum was collected and tested by immuno
precipitation to verify antibody production. Four days after the fourth injection, hybridoma clones were produced as described previ-
ously (32). Hybridoma supernatants were first analyzed by flow cytometry
followed by immunoprecipitation using surface-biotinylated MDA-
MB-435 cell lysates as described above.

Amino-terminal Sequencing of NAG-2 Antigen—MDA-MB-435 cells (5 g) were lysed in 500 ml of buffer containing 1% n-octyl glucoside, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 2 mM phenylmethyl-
sulfonyl fluoride, 15 μg/ml aproptinin, and 60 μg/ml leupeptin. To re-
move background bead-binding material, the lysate was sequentially preincubated with protein A-Sepharose and Sepharose beads conju-
gated with irrelevant mouse mAbs. The precleared lysates were incub-
ated with anti-NAG-2 mAb-conjugated Sepharose beads packed in a
2-ml column. After washing the column, the NAG-2 protein was eluted using 50 mM glycine, pH 3.0, and the fractions were immediately neutral-
ized with 0.1 volume of 1 M Tris-HCl, pH 9.0. Eluted fractions were analyzed on a SDS-PAGE, and the fraction containing the NAG-2 antigen was determined by silver staining. Larger quantities of this fraction were then subjected to SDS-PAGE, and proteins were transferred to a
polyvinilidene difluoride membrane. The 30-kDa band corresponding to the NAG-2 protein was visualized by Ponceau S staining, excised from the membrane, and amino-terminal sequencing was carried out using an Applied Biosystems 470A gas-phase sequenator equipped with a 1-μA phenylhydantoin amino acid analyzer (Harvard microse-
quence facility, Cambridge, MA).

Construction and Screening of cDNA Expression Library—Poly(A)⁺ RNA was isolated from MDA-MB-435 cells, and double-stranded cDNAs were synthesized using Copy Kit (Invitrogen, San Diego, CA). To facilitate the subcloning procedure, BstXI adapters were placed on the 5’-end of cDNAs. After size fractionation by gel electrophoresis, cDNAs of 0.7–2.0 Kb were excised and ligated into pCDM8 expression vectors (Invitrogen). Then MC1061/P3 bacterial cells were transformed with ligated cDNA library and plated to 54 plates at a density of 1,900 clones/plate. Amplified cDNAs (54 separate pools) were collected and purified using QIAGen Plasmid Purification System (Qinggen, Chat-
sworth, CA). For screening, CHO/P cells (6 × 10⁶/well) were grown on 24-well culture plates and then transiently transfected with each pool of amplified cDNAs using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. After incubation for 18 h, transfected CHO/P cells were stained with anti-NAG-2 mAb, fixed with methanol, and incubated with peroxidase-conjugated goat anti-
mouse IgG (Sigma). Positive cells were visualized using Immunopure Metal Detection Kit (DAB Substrate Kit, DAKO) with light microscopy. Positive cells of cDNA yielding one or more NAG-2-positive CHO/P cells were further subfractionated three more times, until a single clone confer-
ing NAG-2 staining was identified.

DNA Sequencing and Analysis of NAG-2—Both strands of NAG-2 cDNA were sequenced by Sanger’s sequencing method using dye-la-
beled dideoxy nucleotides as terminators. Samples were analyzed on an Applied Biosystem 373A automated DNA sequencer (33). Protein data base searches were carried out using BLASTP via the NCBI BLAST
network service (34). Hydrophobicity plot analysis was performed by the Kyte and Doolittle method (35) with a window of seven residues
using the computer software DNA Strider. Multiple sequence align-
ments and similarity calculations were performed with the extended
GCG sequence analysis software package (36).

Flow Cytometry—Cells were incubated with saturating concentra-
tions of primary mouse mAbs for 45 min at 4 °C, washed twice, and then labeled with fluorescein isothiocyanate-conjugated goat anti-mouse immu
noglobulin. Stained cells were analyzed on a FACScan (BectonDick-
ton, Mountain View, CA).

Northern Blotting—NAG-2 cDNA insert was excised from pCDM8 vector with XhoI and labeled with [α-32P]dCTP using RadPrime DNA Labeling System (Life Technologies, Inc.). Northern blot filters of multiple human tissues (CLONTECH, Palo Alto, CA) were hybridized with the labeled probe according to the manufacturer’s instructions. The probe was stripped off by boiling the filter in sterile H₂O containing 0.5% SDS and rehybridized with a [32P]-labeled human β-actin cDNA. Radioactive bands were detected by autoradiography.

Immunohistochemistry—Fresh tissue was obtained from material
submited to the Department of Pathology, Washington University School of Medicine, St. Louis, MO. The tissue was embedded in OCT compound (Miles Laboratory, Elkart, IN), snap-frozen in liquid nitrogen-cooled isopentane, and stored at −70 °C. Frozen sections (6 μm thick) were fixed in acetone and held at −20 °C before staining with anti-NAG-2 antibody and detection with biotinylated anti-mouse IgG and avidin-biotin-peroxidase complex (Vector, Burlingame, CA), as de-
scribed previously (37). Sections were counterstained with methyl green.

RESULTS

Production of mAbs against Protein Complexes Immunopre-
cipitated by Anti-CD81 mAb—To identify new membrane anti-
gens that associate with TM4SF proteins, we have utilized a monoclonal antibody generation and selection strategy, similar to that described in the accompanying paper (38). Beads coated with anti-CD81 mAb were incubated with MDA-MB-435 cell lysate under non-stringent detergent conditions, and then the isolated bead-protein complexes were used to immunize mice. As shown (Fig. 1, lane c) the resulting mouse immune serum immunoprecipitated a protein co-migrating with CD81 (arrow), as well as several additional biotinylated proteins from MDA-
MB-435 cells. Most of these proteins were not precipitated by preimmune serum (lane d). The biotinylated proteins directly immunoprecipitated by mouse sera (lane c) using stringent detergent conditions (1% Brj 96, 0.2% SDS) closely resemble those indirectly co-precipitated by an anti-CD81 mAb (lane a) under nonstringent (Brij 96) conditions. Notably, a control experiment (lane b) shows that anti-CD81 mAb precipitated mostly CD81 under stringent conditions. From these results we concludce that the mouse was adequately immunized to make antibodies directly recognizing CD81-associated cell-surface proteins.

After four injections with CD81 complexes, 560 hybridomas
were prepared, and supernatants were tested by flow cytom-
etry to show that 35 hybridoma clones produced antibodies to cell-surface molecules on MDA-MB-435 cells. Secondary screening by non-stringent immunoprecipitation then was used to confirm whether these 35 mAbs indeed co-precipitated a protein resembling CD81. Immunoprecipitations from four rep-
resentative antibodies are shown in Fig. 2. Under nonstringent conditions, these four antibodies (lanes c, e, g, i) as well as anti-CD81 (lane a) each precipitated a similar protein pattern that included a ~22-kDa component co-migrating with CD81.

In contrast, under stringent conditions mAb M38 yielded only CD81 (lane b). Ab 4D5 yielded a CD9-like protein (lane f) and mAb 8H6 recognized solely an α6 integrin-like protein (lane j).

The antigens directly recognized by mAb IES (lane d) and 5C12 (lane h) were not obvious, possibly because these proteins are not very well labeled with biotin. A weak nespecific 70-kDa band appeared in all lanes upon long exposure. Interestingly, immunoprecipitation data under stringent conditions indicated
that none of the 35 selected mAbs recognized CD81 itself.

Notably, mAb 1E5, along with 29 other mAb, selectively stained CD63-positive CHO transfectants, thus establishing conclusively that these 30 mAbs recognize CD63. Similarly, mAb 4D5 and another mAb selectively stained CD9-transfected HT1080 cells, and mAb 8H6 selectively stained α6-transfected K562 cells, thus confirming CD9 and α6 assignments from Fig. 2. Two other antibodies (5C12 and 2E12) failed to stain hamster or mouse transfectants expressing human α3, α6, or β1 integrin subunits or either of the TM4SF protein transfectants.

Competitive antibody binding assays revealed that the two antibodies of unknown specificity seemed to recognize the same or overlapping antigenic epitopes on the surface of MDA-MB-435 cells (data not shown). This putative novel antigen was named NAG-2.

Characterization of NAG-2 Protein—Although not labeled with biotin (Fig. 2, lane h), the NAG-2 protein could be 125I-labeled and immunoprecipitated under stringent conditions from MDA-MB-435 cells as a somewhat diffuse protein band of 28–35 kDa (Fig. 3). This band was not seen in CD81 (M38), integrin β1 (TS2/16), or negative control (P3) immunoprecipitations. To characterize further NAG-2, ~23 pmol of protein was purified from MDA-MB-435 cells using an anti-NAG-2 immunoaffinity column. Amino-terminal analysis of the purified material yielded a “RA-LQAVKY” sequence that was not present in the GenBank database.

Cloning of NAG-2 cDNA—A cDNA expression library was prepared from MDA-MB-435 cells and transiently transfected into CHO/P cells for screening, based on cell-surface staining with anti-NAG-2 mAb. One of 54 separate pools of clones yielded a few visibly stained CHO/P cells (out of 6 × 10⁶ cells plated). After three more rounds of subfractionation, a single clone was isolated that was capable of conferring anti-NAG-2 staining upon transfection into CHO/P cells.

The entire cDNA sequence of NAG-2 and its corresponding amino acid sequence are shown in Fig. 4A. It contains a 5'-untranslated sequence of 104 base pairs, a single extended open reading frame of 714 base pairs, followed by 540 base pairs of 3'-untranslated region containing a polyadenylation signal. The first ATG codon of the open reading frame is within
Novel TM4SF Protein in TM4SF-Integrin Complexes

FIG. 4

Amino acid number

Hydropathy

Cysteine sequences

Consensus

Consensus

Consensus

Consensus

Consensus

Consensus

Consensus
FIG. 5. Immune complex phosphatase assays. The indicated mAbs were used to immunoprecipitate proteins from 1% Brij 96 lysates (each from $5 \times 10^6$ MDA 435 cell equivalents). Immune complexes were then analyzed for phosphatase activity as described previously (40) in two separate experiments (A and B). Activity was measured as absorbance at 405 nm of p-nitrophenol product derived from p-nitrophenyl phosphate. As determined by flow cytometry, CD109, CD63, and NAG-2 were present on the surface of MDA 435 cells at comparable levels (40–50 mean fluorescence intensity units), whereas CD81 and CD9 were at ~200 mean fluorescence intensity units. CD63 is also expressed in intracellular granules (16).

a Kozak consensus translation initiation sequence (39). The deduced 238 amino acids include the partial sequence obtained by amino-terminal analysis (underlined). The predicted protein molecular mass (26,177 Da) is slightly smaller than that observed in Fig. 3. Potential N-glycosylation sites are located at residues 152 and 161. A hydrophobicity plot revealed four highly hydrophobic domains, each sufficiently long to span cellular membranes (Fig. 4B). In addition, BLASTP searching revealed sequence similarity between the NAG-2 protein and TM4SF proteins, with CD53 (a TM4SF protein expressed on leukocytes) showing the most similarity (51% similarity, 34% identity). The NAG-2 protein sequence was aligned with known human TM4SF proteins (Fig. 4C). For NAG-2 and other TM4SF proteins, conserved sequences are mostly within the putative transmembrane domains. In contrast, the extracellular domains are more divergent in terms of length, sequence, and degree of glycosylation. However, NAG-2 does contain four characteristic cysteine residues, within the large extracellular domain between transmembrane domains III and IV, that are highly conserved among nearly all TM4SF proteins. In addition, a search of the dbEST sequence data base revealed five overlapping expressed sequence tags, from which a complete putative murine NAG-2 sequence was constructed (Fig. 4D). Notably, the level of identity (95%) between the murine and human protein sequences is unusually high. Other TM4SF proteins (e.g. CD53, CD82, CD37, and CD63) typically show 82–83% identity between human and mouse proteins.

The TM4SF proteins CD53 and CD63 were previously shown to be associated with phosphatase activity (40). Here we carried out similar phosphatase assays, utilizing immune complexes from MDA 435 cells lysed in 1% Brij 96. Activity was determined by measuring the conversion of p-nitrophenyl phosphate to the yellow-colored p-nitrophenol product. As indicated (Fig. 5, A and B), phosphatase activity was readily detected in association with CD63 immune complexes in two separate experiments. However, there was minimal phosphatase activity associated with NAG-2 immune complexes (obtained using two different antibodies). Likewise, the activity associated with CD9 or CD81 was not appreciably above the negative control activities seen with mAb P3 or anti-CD109 mAb.

Expression and Distribution of NAG-2—Flow cytometric analysis revealed that NAG-2 protein is expressed most strongly on a human melanoma cell line (LOX), a fibrosarcoma line (HT-1080), and the breast carcinoma line (MDA-MB-435) and shows variable levels on other human sarcoma and carcinoma cell lines derived from various tissues. Also, NAG-2 showed little or no expression on several hematopoietic cell lines and was absent from normal peripheral blood T-cells and platelets (Table I). Northern blot analysis showed that a 1.5-kb transcript of NAG-2 is present in all human tissues analyzed with the exception of brain (Fig. 6). Expression is especially strong in spleen, colon, and pancreas. Heart and skeletal muscle have an additional 6.5-kb NAG-2 transcript.

Frozen section immunohistochemistry was carried out to characterize further the distribution of NAG-2 protein. The NAG-2 protein was widely expressed in all tissues evaluated but was present in a restricted repertoire of cell types (Table II). High levels of NAG-2 were observed on vascular endothelial cells and fibroblasts in all tissues. The spleen, composed of a meshwork of branching vascular cords and sinusoids, expressed the highest levels of NAG-2 (Fig. 7A). In contrast, red pulp cords, thin walled vessels, lymphocytes of the lymphoid follicles, and periarterial lymphoid sheaths were entirely negative. Also, spleen showed greater NAG-2 expression in veins, sinusoids, and small arteries, compared with thick walled arteries (Fig. 7A).

Expression of NAG-2 in tonsil is also complex. The non-keratinizing stratified squamous epithelium of the tonsil showed very low levels of NAG-2, with expression limited to the basal cell layer with formation of a discrete band at the basement membrane. Lymphoid cells of the lymphoid follicles and the interfollicular T-cell zones were also negative, whereas the delicate, dendritic, reticulum cells of the follicles showed intermediate to strong staining (Fig. 7B). Also, fibroblasts and endothelial cells within the medullary cords and interfollicular zones showed intermediate NAG-2 staining.

In thymus, lymphoid cells failed to express NAG-2, but thymic lobules were demarcated by strongly positive stromal cells.

FIG. 4. Nucleotide and deduced amino acid sequence of NAG-2. A, nucleotide and amino acid numbers are indicated to the right of the sequence. The region corresponding to the peptide sequence obtained from amino-terminal sequencing is underlined. Two potential N-glycosylation sites are circled. A predicted polyadenylation signal is doubly underlined. B, a hydrophobicity plot of the deduced amino acid sequence was determined by the Kyte and Doolittle method with a window of seven residues. Four putative transmembrane domains are numbered above the plot. C, the NAG-2 protein was aligned with other human TM4SF members using PILEUP and PRETTYPLOT programs in the extended GCG package, with minor additional adjustments to improve alignment. The amino acids that are identical in at least seven proteins are boxed, and consensus amino acids are shown below the sequences. Four putative transmembrane domains are indicated by thick bars. Four highly conserved cysteines in the extracellular domain between transmembrane III and IV are marked by asterisks. Similarity values relative to the NAG-2 sequence (shown in parentheses) were calculated using the OLDDISTANCES program in the GCG package. D, a complete sequence for putative mouse NAG-2 protein was deduced from five overlapping expressed sequence tags (accession numbers in dbEST: AA002806, AA138105, AA107941, AA044511, and D18312). Regions of identity are boxed; cysteines characteristic of most TM4SF proteins are marked with asterisks; the four putative transmembrane regions are overlined, and two potential N-glycosylation sites are underlined.
TABLE I
Expression of NAG-2 protein in cultured human cells

| Cells               | Fibrosarcoma | HT1080 | Ramos | Rhabdomyosarcoma | RD |
|---------------------|--------------|--------|-------|-----------------|----|
| B-cells             |              |        |       |                 |    |
| Raji                |              |        |       |                 |    |
| JY                  |              |        |       |                 |    |
| Ramos               |              |        |       |                 |    |
| T-cells             | Breast carcinomas | MDA-MB-435 | MDA-MB-231 | Lung carcinoma |   |
| Jurkat              |              |        |       |                 |    |
| Molt-4              |              |        |       |                 |    |
| peripheral blood    |              |        |       |                 |    |
| Promyelocytic       |              |        |       |                 |    |
| K562                | ++           | A549   |       |                 |    |
| Platelets           |              |        | Colon carcinomas | CCL 221 |   |
| Melanoma            | +++          | CCL 227 | +     |                 |    |
| LOX                 |              |        |       |                 |    |
| Neuroblastomas      |              | CCL 228 | +     |                 |    |
| IMR-32              | +            |        |       |                 |    |
| SK-N-SH             | ++           | A431   |       |                 |    |

TABLE II
Histologic distribution of the NAG-2 protein

| Cell type               | Intensity |
|-------------------------|-----------|
| Fibroblasts             | 3+        |
| Endothelial cells       | 3–4+      |
| Smooth muscle           | 0–1+      |
| Cardiac muscle          | 1–2+      |
| Neurons                 |           |
| Cerebral cortex         | 0         |
| Cerebellum              | 0         |
| Ganglion cells          | 2+        |
| Lymphocytes             |           |
| Tonsil                  | 0         |
| Spleen                  | 0         |
| Thymus                  | 0         |
| Dendritic cells         | 3–4+      |
| Follicular (tonsil & spleen) |       |
| Mesothelial cells       |           |
| Colon                   | 4+        |
| Thymus                  | 4+        |
| Epithelium              |           |
| Breast                  | 0–1+      |
| Colon                   | 0–1+      |
| Keratinizing (skin)     | 0–1+      |
| Non-keratinizing (tonsil) | 0–1+  |
| Liver                   | 1+        |

Occasional dendritic reticulum cells within the center of thymic lobules were also weakly positive (Fig. 7C). Columnar epithelium of the colon lining the crypts and smooth muscle of the muscularis mucosa weakly expressed NAG-2, but the thick smooth muscle bundles of the muscularis externa were entirely negative (Fig. 7D). Also, mesothelial cells lining the serosal surface were strongly positive, and fibroblasts, endothelial cells, and occasional ganglion cells within the colonic wall also expressed NAG-2.

In other tissues the epithelium of breast ducts and the terminal duct units showed very low levels of NAG-2. Brain including cortical tissue and cerebellum failed to express NAG-2, whereas the parenchymal cells of the liver and heart both expressed intermediate levels. In skin, NAG-2 staining was similar to that seen in tonsil. Stratified squamous epithelium showed only low levels, except for a thin band at the basement membrane (as in tonsil).

NAG-2 Associates with CD9, CD81, and α3, α5, and β1 Integrins—Since the anti-NAG-2 mAb 5C12 was obtained following immunization with CD81-protein complexes, we assumed that NAG-2 is a component of TM4+TM4 and possibly also TM4+TM4-integrin complexes. To confirm this, re-immunoprecipitation experiments were carried out (Fig. 8). Anti-NAG-2 immunoprecipitation complexes were prepared from MDA-MB-435 cells under nonstringent conditions, and then complexes were dissociated using 0.2% SDS. From the eluate, TM4SF proteins, including CD9, that could be reprecipitated. Likewise, α3, α5, and β1 but not α2 or α5 integrins could be reprecipitated (Fig. 8B, lanes g–k). Control experiments demonstrated that all of the proteins analyzed could be directly immunoprecipitated in comparable abundance (lanes d–f and l–p). Reciprocal re-immunoprecipitation results confirmed the pattern of NAG-2-associated proteins. NAG-2 could be reprecipitated (Fig. 8C) from complexes initially isolated using anti-CD9 (lane q), −CD81 (lane r), −α3 (lane u), −α5 (lane w), or −β1 antibodies (lane x) but not from CD109 (lane s), α2 (lane t), or α5 (lane v) complexes. Also immunoprecipitations of NAG-2 (but not CD109) yielded TM4SF proteins, including CD9, that could be directly immunoblotted (not shown). To demonstrate further NAG-2 association with another TM4SF protein, immunofluorescent staining was carried out. As indicated by double staining in Fig. 9, CD81 and NAG-2 were co-localized in focal complexes at the periphery of spread HT1080 cells and in intracellular vesicles. In contrast, MHC class I protein showed a distinct and more evenly distributed pattern of staining.

DISCUSSION

Here we have identified and characterized a new protein, NAG-2, that interacts with integrins (α3β1 and α5β1) and TM4SF proteins (CD9 and CD81). The NAG-2 protein is itself a new TM4SF member, and as seen for other TM4SF members (2), NAG-2 did not interact with α2β1 or α5β1. Two mAbs
against NAG-2 protein were selected after immunization with integrin-CD81 protein complexes. Notably, of all selected mAbs (total 35 mAbs), we obtained no antibodies to any other cell-surface proteins (besides integrins or TM4SF proteins) even though integrin-TM4SF protein complexes contain additional cell-surface components. These results reinforce the existence of integrin-TM4 and TM4TM4 complexes, as previously demonstrated by experiments involving reciprocal co-precipitation, cell-surface cross-linking, and immunofluorescence co-localization (6, 7, 15, 16). Furthermore, these results suggest that integrins and TM4SF proteins may be dominant members of these complexes, and perhaps also the most immunogenic of the proteins present in the complexes. Thus, another strategy might be needed to allow characterization of other components in these complexes.

With the addition of NAG-2, mammalian TM4SF proteins now include at least 11 clearly identifiable members (listed in Fig 5C), with 6 more distantly related members (peripherin/RDS (41), rom-1 (42), L6 (43), uroplakins Ia and Ib (44), and il-TMP (45)). Another 3 TM4SF proteins have been identified in other organisms, including Drosophila (46) and worms (47, 48).

The NAG-2 protein has all key structural features of classical TM4SF proteins. These features include the following: four highly conserved hydrophobic domains, presumed to span the lipid bilayer; short regions (5–14 amino acids), predicted to be cytoplasmic domains at the amino and carboxyl termini; hydrophilic domains between transmembrane I and II and between III and IV that correspond to a predicted small extracellular loop (20–27 amino acids) and a larger extracellular loop (75–130 amino acids); a Cys-Cys-Gly motif and two other cysteines in the large extracellular loop that are highly conserved in most TM4SF proteins. In addition, the third putative transmembrane domain of NAG-2 contains a glutamate residue (Glu-97) characteristic of many TM4SF proteins.

The size of NAG-2 (26,177 Da), predicted from the cDNA sequence, is slightly smaller than the actual size seen in SDS-PAGE (28–35 kDa). However, a pulse-chase experiment using radiolabeled methionine showed a precursor 26-kDa form maturing to a more diffuse 30-kDa protein within an hour. This maturation is most likely due to N-glycosylation of one or both of the possible N-glycosylation sites in NAG-2. Northern blot analysis revealed that in addition to the major NAG-2 1.5-kb transcript detected in various epithelial and mesenchymal tissues, skeletal muscles and heart also express a 6.5-kb message. It is not known whether this long transcript (unique among TM4SF members) represents an alternatively spliced variant transcribed from a single NAG-2 gene or is a product of separate gene. In this regard, the mouse genome contains two separate genes encoding CD63 (49).

At the protein level, NAG-2 was detected on a variety of cell lines and within different tissues, thus suggesting that NAG-2, along with other TM4SF members with wide tissue distribution (e.g. CD9, CD63, and CD81), may be of general physiological importance. On the other hand, NAG-2 is generally expressed on a more limited number of cell types within each tissue, compared with the previously described distribution of CD9, CD63, and CD81 (6). The extensive appearance of NAG-2

\[ I. Tachibana, J. Bodorova, F. Berditchevski, M. M. Zutter, and M. E. Hemler, unpublished observations. \]
protein on endothelial cells and cells proximal to basement membrane somewhat overlaps with the distribution pattern for the α3β1 integrin (6) and thus is consistent with their co-association. Notably, both Northern blot analysis and immunohistochemical staining failed to detect appreciable expression of NAG-2 in normal brain tissue. However, NAG-2 was expressed at moderate levels on cells of neural crest origin, including the neuroblastoma cell lines, IMR-32 and SK-N-SH, as well as mature ganglion cells in the colon.

Functions of NAG-2-integrin and NAG-2 TM4SF protein complexes are currently unknown. It is unlikely that interaction with NAG-2 (and other TM4SF proteins) is required for α3β1 (or α6β1)-mediated cell adhesion. Indeed, the breast carcinoma line MDA-MB-231, which expresses only negligible amount of NAG-2 (Table I), is fully capable of adhering to laminin-5 in an α3β1-dependent manner (data not shown). Instead, integrin-NAG-2 complexes may play an important role in triggering post-adhesion signaling events. In this regard, membrane complexes containing TM4SF proteins can also include various signaling proteins. For example, the CD21-CD19-CD81(TAPA-1) complex in B-cells is associated with phosphatidylinositol-3 kinase (50), whereas CD63/β2-integrin complexes on neutrophils may contain Src family tyrosine kinases (51). Also, leukocytes CD53 and CD63 may associate with protein phosphatase activity (40), and in multiple cell types, integrin-NAG-2 TM4SF protein complexes contain associated phosphati-
dylositol-4 kinase activity (20). However, when analyzed in several cell lines we have not found that any of these enzymatic activities associate with NAG-2 (Fig. 5).3

Integrin-NAG-2-other TM4SF protein complexes possibly could be important during cell migration. For example, we found that NAG-2 protein, like CD81, is specifically localized in focal complexes at the periphery of lamellipodial protrusions, as previously seen for TM4SF proteins (e.g. CD81, CD63, and CD9) and the integrins αβ and αβ (20).2 Since lamellipodial extensions are typically associated with cell motility, it is possible that activity of integrin-NAG-2 protein complexes may contribute to a migratory phenotype of cultured tumorigenic cells.

In conclusion, we have established that major components in CD81 protein complexes are other TM4SF proteins, as well as integrins. In addition, we have discovered and characterized a novel TM4SF protein called NAG-2. The widespread distribution of NAG-2 protein on discrete cell types in many tissues and organs and its unusually high sequence conservation between human and mouse are consistent with it having an important functional role.

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