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v-*rel* Induces Ectopic Expression of an Adhesion Molecule, DM-GRASP, during B-Lymphoma Development

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In an effort to identify aberrantly expressed genes in v-*rel*-induced tumors, monoclonal antibodies were developed that reacted selectively with avian B-cell tumors. One antibody, HY78, immunoprecipitated a 120-kDa glycoprotein (p120) from cells that express v-*rel*. N-terminal amino acid sequencing of p120 identified a 27-amino-acid sequence that is also present in DM-GRASP, an adhesion molecule belonging to the immunoglobulin superfamily. Evidence from tissue distribution, immunological cross-reaction, PCR amplification, cDNA cloning, and DNA sequence shows that p120 is indeed DM-GRASP. Northern (RNA) analysis using a probe from the DM-GRASP gene identified a 5.3-kb transcript in mRNA from bursa, thymus, and brain as well as from v-*rel*-induced B-cell lymphomas but not from bursal B cells. The induction of this protein by v-*rel* during the development of bursal B-cell lymphomas appears, therefore, to be ectopic in nature. Overexpression of v-*rel* or c-*rel* in chicken embryonic fibroblasts, B-cell lines, and spleen mononuclear cells induces the expression of DM-GRASP. The ratio of DM-GRASP to v-Rel was fivefold higher than that of DM-GRASP/c-Rel in a B-cell line, DT95. Interestingly, the presence of HY78 antibody inhibits the in vitro proliferation of v-*rel*-transformed cells but not cells that immortalized by *myc*. These data suggest that DM-GRASP is one of the genes induced during v-*rel*-mediated tumor development and that DM-GRASP may be involved in the growth of v-*rel* tumor cells.

Increasing evidence demonstrates that alterations in host gene expression by viral oncogenic proteins are important in transformation by oncogenic retroviruses. Several mechanisms are apparently involved in this process. Nuclear oncoproteins, such as v-Myb, v-Fos, and v-Jun, bind to their cognate DNA sequences and directly regulate gene expression (39, 42). Other oncoproteins that reside in the cytoplasm or on the cell membrane, e.g., v-Src or v-Ras, alter gene expression through intracellular signaling pathways (40, 68). The v-*rel* oncogene, present in the avian reticuloendotheliosis virus, strain T (REV-T) (34), encodes a protein that localizes in both the cytoplasm and the nucleus in transformed cells and can modulate transcription (13, 29). $p59^{v\text{-}rel}$ belongs to the NF-κB/Rel family, members of which contain a conserved 300-amino-acid stretch in their N-terminal regions (28). This family, which includes the p50, p52, p65, and RelB subunits of NF-κB, as well as the proto-oncogene c-*rel*, regulates genes containing κB elements (2, 30, 47). The κB consensus sequence has been identified in promoters and enhancers in a variety of cellular genes, including immunoreceptors, such as immunoglobulin κ light chain (67), interleukin-2 receptor (IL-2R) (70), major histocompatibility complex class I (MHC class I) (3), and MHC class II (11); cytokines, such as interleukin-2 (27), interleukin-6 (52), and β-interferon (45); growth factors, such as granulocyte-macrophage colony-stimulating factor (66); and surface molecules that function in extracellular signaling and cell adhesion, such as intercellular cell adhesion molecule 1 (ICAM-1) (73) and vascular cell adhesion molecule 1 (VCAM-1) (69). These data suggest that NF-κB/*rel* transcription factors play a central role in the regulation of genes that control cell communication, differentiation, and proliferation in the immune system.

v-*rel* transforms avian hematopoietic cells with high efficiency (46). The resulting tumors bear markers characteristic of B cells, T cells, or cells of the myeloid lineage (6, 7, 77). v-*rel* can also transform chicken embryonic fibroblasts (CEF) as evidenced by an altered cellular morphology (24, 48, 50). The mechanism of v-*rel*-mediated transformation is presumably related to its effects on transcriptional activity. Although v-*rel* can activate viral gene expression in a species-specific and promoter-specific manner (26), the transactivation activity of v-*rel* did not appear to be directly related to its ability to induce tumors, on the basis of an in vitro transient expression assay (31, 41). Instead, v-*rel* was shown to inhibit the transactivation mediated by p50, p65, and c-*rel* by targeting κB elements (4, 5, 38), and this inhibitory activity was correlated with its transforming ability (5, 61). Recently, it was observed that transcription modulation by v-*rel* was also affected by the differentiation status of the cells: v-*rel* was transactivating in an undifferentiated cell line and became transrepressing when the cells underwent differentiation (74). Also, using a v-*rel* estrogen-receptor fusion protein, Boehmelt et al. identified two genes, MHC class I and high mobility group protein 14b (HMG-14b), whose expression was upregulated by v-*rel* on the transformed cells (12), although no data were presented to show that these two genes play a role in tumor development. More recent data also demonstrated that v-*rel* induced the expression of MHC class I, MHC class II, and IL-2R in tumor cells transformed either in vivo or in vitro (35). Although c-*rel* also induced these genes, the level of induction by c-*rel* was substantially weaker than that of v-*rel*. Studies of the c-*rel*/v-*rel* chimeras demonstrated that the relative levels of induction of these three proteins by the chimeras correlated with their transforming potentials (55). Taken together, these results suggest that v-*rel* transforms

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cells by disrupting the normal transcription balance by acting as both a transactivator and a transrepressor.

In this study, we used a monoclonal antibody, HY78, which was isolated following a subtractive procedure, to identify a gene product that appears to be ectopically induced by v-*rel* in B-cell lymphomas. This gene, DM-GRASP, belongs to the immunoglobulin superfamily and encodes an adhesion protein that may be involved in the development of both neuronal and hematopoietic tissues (14, 20). We also showed that HY78 monoclonal antibody selectively inhibits the growth of v-*rel*-tumor cells in culture.

MATERIALS AND METHODS

Plasmids. pREV-v-*rel* is a REV-T-based vector containing the entire v-*rel* gene (17). pREV-c-*rel* is a similar construct containing the complete coding region from a chicken c-*rel* cDNA (15). These constructs have been previously described (55). pCSV11S3 contains an infectious provirus of the chicken syncytial virus (CSV) which was derived from the 11S3 cell line (8) and transformed by REV-T using CSV as a helper virus. pCMV-GRASP is a cytomegalovirus-based vector containing the entire coding region from a DM-GRASP cDNA (14).

Antibodies. The following monoclonal antibodies used in this study recognize previously described antigens. The antibodies with a prefix HY were generated in our laboratory: HY83 (anti-CSV) (35), HY78 (anti-p120), and HY82 (anti-p75) (37). 3C1 (anti-v-Rel and c-Rel) (22) was provided by H. R. Bose (University of Texas at Houston). DM1 and DM2 (anti-DM-GRASP) (14) were provided by S. Chang (University of Pennsylvania). Anti-HNK-1 was obtained from the American Type Culture Collection (58).

Cell lines and viral infection. DT40, DT45, and DT95 are avian leukosis virus-induced bursal lymphoma cell lines (1). RP9 is a cell line similar to DT40 (56). RP13 is a B-cell line derived from CSV-induced lymphoma (54). BM2 is an avian myeloblastosis virus-transformed cell line (51). S2A3 is a REV-T nonproducer cell line derived from in vitro infection of chicken spleen cells (46). 30B3 and 27L2 are B-cell lines derived from REV-T/CSV-induced v-*rel*-driven lymphoma (6). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% tryptose phosphate broth, 5% calf serum, and 5% chicken serum. Stocks of REV-T/CSV viruses were prepared by infecting S2A3 with CSV. The medium was harvested and stored at −70°C. Infection of DT40 cells was done by incubation of the cells with REV-T/CSV viruses at 37°C for 60 min followed by plating the cells onto tissue culture plates. DT40(CSV) and DT40(T) cell lines were isolated by the limiting dilution method and immunofluorescence staining for the CSV antigen and v-*rel* protein.

Transfection and cocultivation. DNA transfection was done by using a modified calcium phosphate precipitation method (16). Secondary CEF were used for transfection with 20 μg of DNA of defective virus (pREV-v-*rel* or pREV-c-*rel*) and 2 μg of helper virus (pCSV11S3). Approximately 7 days after transfection, confluent CEF were cocultivated with suspension cells. Cells (5×10^6) were applied to a 10-cm CEF plate and cocultivated for 1 h. Cells were collected by washing off gently from CEF and cultured on a plastic plate. Samples were collected at indicated time points for cell staining and protein and RNA analyses.

In vitro cell proliferation assay. Cells (10^5) in 1 ml of media containing 0.5, 1, 5, 10, and 20 μg of antibodies purified from protein A-Sepharose beads (Pharmacia) were cultured for 2 to 3 days. The cells were then counted and resuspended exactly as described for day 0. The total cell number was calculated from each time point.

Generation of monoclonal antibodies. The immunization procedure was as described by Huffnagle et al. (36). DT40(T) cells (10^7) were injected intraperitoneally into 4- to 6-week-old BALB/c mice three times with a 3-week interval between injections. Immunized mice were sacrificed 3 days after the final boost, and splenic cells were fused with a myeloma cell line, Sp2/0, by using a stirring method (32). Prior to screening, DT40(T) and DT40(CSV) cells (2×10^5 per well) were fixed on a 96-well plastic plate (Becton Dickinson) by methanol-acetone (1:1) for 5 min at room temperature. The plastic plate had been coated with poly-L-lysine at 20 μg per well overnight at 4°C. Fifty microliters of hybridoma supernatant was added to each well and incubated at room temperature in a humidified atmosphere for 1 h. The plates were washed three times with phosphate-buffered saline (PBS). The second antibody, biotinylated goat anti-mouse immunoglobulin (Southern Biotechnology), was then added to each well (50 μl per well) at a 1:2,000 dilution in 1% nonfat milk–PBS. After incubating at room temperature for 40 min, the plates were washed as described above and 50 μl of 1:3,000 diluted streptavidin-alkaline phosphatase (Boehringer Mannheim) was applied to each well. The plates were incubated for another 40 min and washed twice with PBS, once with washing buffer (10 mM KH_2PO_4, pH 6.5, 0.5 M NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% bovine serum albumin), and once with predetection buffer (10 mM diethanolamine, pH 9.5, 0.5 mM $MgCl_2$). The reaction was developed with 50 μl of predeveloping buffer containing 50 μg of *p*-nitrophenyl phosphate (Pierce). Hybridoma supernatants were first screened on DT40(T) cells. Positive supernatants were then screened on DT40(CSV) cells. Approximately 4,000 hybridomas (four fusions) were raised. Approximately 400 reacted with DT40(T). We obtained four hybridomas that produced monoclonal antibodies reacting with DT40(T) but not with DT40(CSV).

Cell labeling, immunoprecipitation, and Western blot (immunoblot). Cells were washed once in PBS and starved in methionine-free DMEM containing dialyzed 5% fetal calf serum–5% chicken serum for 2 h at 37°C and 5% CO_2. Cells were then pelleted and resuspended in the above-described medium containing serum plus ^{35}S-methionine (Amersham) at 10^7 cells/600 μCi/0.5 ml of medium and labeled from 1 to 3 h. For the glycosylation assay, cells were labeled under the same conditions as above except that 20 mM 2-deoxyglucose was included in the starvation and labeling medium. After labeling, the cells were washed once with PBS and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium dodecyl sulfate [SDS], 1% Triton X-100, 2 mM EDTA, 1 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride). For immunoprecipitation, cell lysates were cleared by microcentrifugation at 4°C for 20 min. Two microliters of hybridoma ascites or 50 μl of hybridoma supernatants was incubated with lysates prepared from either 1×10^6 labeled ($\sim 2 \times 10^7$ cpm) or 1×10^7 unlabeled cells at 4°C for 2 h. Then, 150 μl of a 25% suspension of protein A-Sepharose beads (Pharmacia) was added to the above lysate, and the mixture was incubated for another 1 h. The beads were pelleted and washed four times with RIPA, boiled with loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.2% bromophenol blue), and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For labeled proteins, the gel was fixed with methanol-acetic acid and stained with Coomassie blue R250. The gel was incubated with 1 M sodium salicylate for 30 min before exposing to X-ray film from 1 day to 1 week. For unlabeled immunoprecipitates, the protein was electrotransferred to a 0.45-μm-pore-size nitrocellulose membrane (Schleicher & Schuell) in 25 mM Tris–125 mM glycine–20% methanol at 100 V for 1 h. The membrane was then blocked with 1% nonfat milk–TBS (TBS is 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). The membrane was incubated with the primary antibody diluted 1:1,000 in TBS for ascites-derived antibodies or 1:10 for tissue culture supernatants for 1 h. After three washes with TBS, the membrane was subjected to a biotinylated straptavidin-alkaline phosphatase protocol similar to the one described for the hybridoma screening except that nitroblue tetrazolium and 5-bromo-4-chloro-3 indolylphosphate toluidinium were used as substrates. Some blots were developed by the horseradish peroxidase-enhanced chemiluminescence method (Amersham). For quantitative analysis, filters were scanned by a Bioimage XRS scanner using computer software for data analysis.

Protein purification and N-terminal amino acid sequencing. p120 was purified by immunoaffinity chromatography. The chromatography was done according to the manufacturer's directions (Pierce). Briefly, 1.5 ml of protein A-Sepharose beads was coated with 2 mg of rabbit anti-mouse immunoglobulin (Pierce) and then reacted with 5 ml of HY78 ascites fluid. The antibodies bound to the protein A beads were cross-linked with 20 mM dimethyl pimelimidate (Pierce) in 0.1 M sodium borate, pH 9.0. 30B3 cells (5×10^9) were lysed in RIPA buffer. The lysate was cleared by centrifugation at 20,000 × *g* for 30 min, then passed through a column containing rabbit antibodies to remove proteins that would bind nonspecifically. The lysate was passed through the immunoaffinity column two times, and p120 was eluted with 50 mM diethanolamine, pH 11.5. Approximately 10 μg of p120 was purified by silver staining of a portion of the eluate on SDS-PAGE. The partially purified p120 was separated by SDS-PAGE and transferred to a 0.45-μm-pore-size polyvinylidene difluoride membrane (Millipore) in 10 mM cyclohexylamino-1-propane-sulfonic acid (Sigma) (pH 11)–10% methanol for 45 min at 90 V. The membrane was stained with 0.1% Coomassie Blue R250. The p120 component was excised. The immobilized protein was then subjected to automated random degradation using an applied amino acid sequencer (model 477A; Applied Biosystems Inc., Foster City, Calif.) with manufacturer's standard chemicals and programming.

RNA preparation and analysis. Total cellular RNA was prepared following the acid-guanidine-thiocyanate-phenol method (18). The poly(A)$^+$ RNA was isolated by oligo(DT)-cellulose followed the manufacturer's directions (Invitrogen). Five micrograms of poly(A)$^+$ RNA was separated on a 0.8% agarose-formaldehyde gel and transferred to an Immobilon-N membrane (Millipore) in 10× SSC buffer (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). The membrane was prehybridized in buffer A (0.5 M sodium phosphate, pH 7.4, 5 mM EDTA, 0.1% bovine calf serum, 0.1% Ficoll, 0.5% SDS) at 68°C for 2 h and then hybridized with [α-^{32}P]dCTP (ICN)-labeled cDNA probes in buffer A plus 100 μg of yeast tRNA per ml overnight. After two washes with 2× SSC–0.5% SDS at 68°C, the membrane was exposed to film at −70°C from several hours to several days with intensifying screens.

cDNA cloning, PCR, and DNA sequencing. A cDNA library was made from DT40(T) mRNA using a Stratagene cDNA kit. The library was screened with anti-p120 monoclonal antibodies or with a 0.7-kb probe from DM-GRASP (*Pst*I-*Eco*RI fragment) following standard protocols (62). Plaques (2×10^6 or 5×10^5) were screened by antibody or an α-^{32}P-labeled DNA probe, respectively. Four oligonucleotides synthesized by the Recombinant DNA Core Facility at West Virginia University were used for PCR amplification. They were designated C1 (5′ AAT ACC ATC CAC GTT CAT GC 3′), C2 (5′ TAT ACC TTC TGT TTC TCC TGG 3′), N1(5′ GTA AAT GCA GTA TAT GGA GAC 3′), and N2 (5′ ACA GAA GAT GAT GTT TCC GAG 3′). By using C1 as a primer, specific cDNA was synthesized from DT40(T) or 30B3 mRNA with a Stratagene cDNA kit, in which 1 μg of poly(A)$^+$ selected RNA was reverse transcribed into cDNA

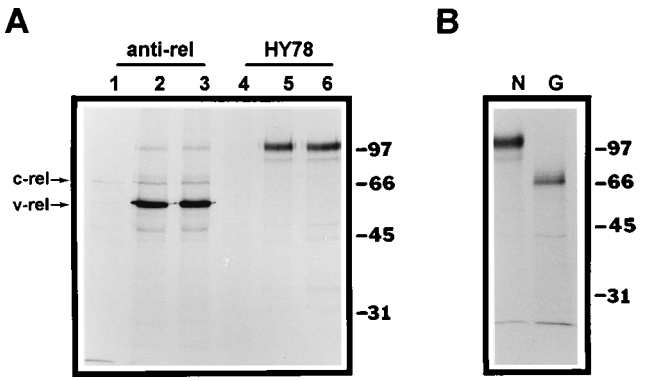

FIG. 1. HY78 monoclonal antibody recognizes a glycosylated protein in cell lines expressing v-*rel*. (A) Cell lysates from ^{35}S-methionine-labeled DT40(CSV) (lanes 1 and 4), DT40(T) (lanes 2 and 5), and 30B3 (lanes 3 and 6) were immunoprecipitated with anti-Rel (lanes 1, 2, and 3) or HY78 (lanes 4, 5, and 6) monoclonal antibodies. Equal amounts of radioactivity (2×10^7 cpm) were used in each reaction. (B) Cell lysates from ^{35}S-methionine-labeled 30B3 with (lane G) or without (lane N) 2-deoxyglucose present as glycosylation inhibitor were incubated with HY78 monoclonal antibody. Equal amounts of radioactivity (3×10^7 cpm) were used in each immunoprecipitation reaction. The immunocomplex was precipitated by protein A-Sepharose beads, separated by 12% SDS-PAGE, and exposed to X-ray film for 3 days. Relative migration sites of marker proteins in kilodaltons are indicated on the right side of the panels.

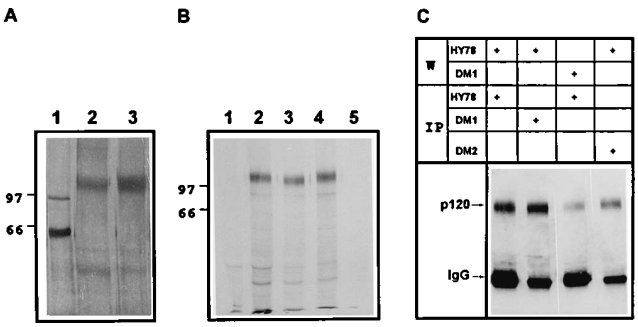

FIG. 2. Immunological detection of p120 and DM-GRASP. (A) Silver staining of immunopurified p120 on an SDS-polyacrylamide gel. Lysate from 5×10^9 30B3 cells was passed through an immunoaffinity column. The protein bound to the column was eluted with 50 mM diethanolamine, pH 11.5. A portion of the eluate was separated by SDS–10% PAGE, and the gel was stained with silver nitrate. Lane 1, molecular marker proteins whose sizes (in kilodaltons) are indicated on the left side of the panel. Lanes 2 and 3, portions from two fractions containing purified p120 eluted from the column. (B) Anti-DM-GRASP and HY78 antibodies recognized proteins that comigrate on SDS-PAGE. Cell lysate from ^{35}S-methionine-labeled DT40(CSV) (lanes 1 and 5) or 30B3 (lanes 2, 3, and 4) was immunoprecipitated by DM1 (lanes 1 and 2), DM 2 (lane 3), or HY78 (lanes 4 and 5) antibodies. The proteins were separated by SDS–10% PAGE and exposed to X-ray film for 2 days. The sizes of the marker proteins (in kilodaltons) are indicated on the left side of the panel. (C) Anti-DM-GRASP and HY78 monoclonal antibodies are cross-reactive. Equal amounts of unlabeled cell lysate from 30B3 were immunoprecipitated by the antibodies indicated in the IP rows and separated by SDS–10% PAGE. The proteins were transferred to a nitrocellulose membrane and stained with the antibodies indicated in the W rows. The filter was developed with a horseradish peroxidase-enhanced chemiluminescence method. p120 and immunoglobulin G are indicated by arrows. IP, immunoprecipitation; W, Western analysis.

in a 20-μl reaction volume. PCR was performed with combinations of C and N primers following the standard protocol (62). The profile of PCR was as follows: 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, for 35 cycles. For DNA sequencing, PCR products were gel purified. Nucleotide sequences of the cDNA clone and the PCR products were determined by the dideoxy chain termination method of Sanger et al. (64) with a sequencing kit from Promega.

RESULTS

Identification of a surface membrane protein induced by v-*rel*. To identify genes whose expression is modulated by v-*rel* during the development of B-cell lymphomas, isogenic paired cell lines were established for the isolation of monoclonal antibodies that specifically recognize antigens on v-*rel*-transformed tumors. DT40, a *myc*-transformed B-cell line, was infected with REV-T(CSV). Two cell lines were produced: DT40(CSV), in which only the helper virus CSV was present, and DT40(T), in which both REV-T and CSV were present. Immunofluorescence analysis directed against CSV-specific viral proteins demonstrated the presence of these antigens in both cell lines; v-*rel* was expressed only in DT40(T). The expression of CSV had no obvious effect on the proliferation or phenotype of DT40 cells. DT40(CSV) grew as nonadherent, single cells indistinguishable from DT40, with a generation time of 8 to 12 h. In contrast, DT40(T) grew as large, semiadherent aggregates of 50 to 200 cells with a generation time of 20 to 24 h. Northern blot analysis demonstrated that the levels of c-*myc* and c-*rel* did not differ significantly among DT40, DT40(CSV), and DT40(T), and only the last expressed v-*rel* (data not shown).

By using a differential screening technique, four monoclonal antibodies were generated that reacted with DT40(T) but not DT40(CSV). One such antibody, HY78, immunoprecipitated a protein with an apparent molecular mass of 120 kDa (p120) from cell lines expressing v-*rel* (Fig. 1A, lanes 5 and 6). Surface staining of 30B3, a v-*rel*-transformed B-cell line, demonstrated that p120 is on the surface of the cell (data not shown). Immunoprecipitation of lysates from ^{35}S-methionine-labeled 30B3 cells treated with the glycosylation inhibitor 2-deoxyglucose showed that the molecular mass was reduced from 120 to 71 kDa (Fig. 1B), indicating that p120 is highly glycosylated. p120 is expressed on all 12 v-*rel*-transformed cell lines examined to date, including lines of B-cell, T-cell, and myeloid origin. In situ immunohistochemistry of livers and bursas from chickens infected with REV-T(CSV) or REV-T(REV-A) showed that p120 was highly induced in both bursal and liver tumors (data not shown; p120 is not found in normal hepatocytes and bursal follicles; see below). p120 is not expressed in three other *myc*-transformed B-cell lines (DT45, DT95, and RP13) but is expressed in one *myc*-transformed B-cell line, RP9, and in the v-*myb*-transformed myeloid cell line, BM2.

N-terminal amino acid sequence of p120 is identical to DM-GRASP. Having failed to isolate clones from a DT40(T) cDNA expression library by using several monoclonal antibodies specific for p120, we purified the protein from 30B3 and DT40(T) cells by immunoaffinity chromatography (Fig. 2A). The partially purified protein was separated by SDS–10% PAGE and transferred to a membrane. The protein was then subjected to an N-terminal amino acid sequence analysis. This analysis produced the following sequence: NH_2-Leu-Tyr-Thr-Val-Asn-Ala-Val-Tyr-Gly-Asp-Thr-Ile-Thr-Met-Pro-X-X-Leu-Glu-Val-Pro-X-X-Leu-Met-Phe-Gly. This sequence was compared with sequences in the GenBank database and was found to be identical to the N-terminal amino acid sequence of DM-GRASP, an adhesion molecule belonging to the immunoglobulin superfamily (14).

DM-GRASP has been studied by a number of laboratories and was named BEN (59) and SC1 (72) as well. To make the description simpler, we arbitrarily used the name DM-GRASP in this paper.

p120 is immunologically cross-reactive with DM-GRASP. Two anti-DM-GRASP monoclonal antibodies, DM1 and DM2, were used in an immunoprecipitation assay with lysate from ^{35}S-methionine-labeled 30B3 cells. Both antibodies precipitated a protein with a size identical to that of p120 (Fig. 2B,

lanes 2 and 3). The specificity of this reaction was tested by cross-blot analysis. Unlabeled 30B3 cells were lysed in RIPA buffer and precipitated with either HY78 or DM1/DM2. The precipitates were separated by SDS–10% PAGE and transferred to a nitrocellulose membrane. The membrane was then blotted with DM1 or HY78 and processed for detection by chemiluminescence. The result demonstrated that HY78 and anti-DM-GRASP antibodies cross-reacted with a protein of the same apparent molecular mass (Fig. 2C). By using DM1 and DM2, the distribution of the protein on tumor cell lines was examined by immunofluorescence microscopy. DM-GRASP was detected on the surface membrane of DT40(T), 30B3, and S2A3 but not on DT40, DT40(CSV), or DT95 (data not shown). The monoclonal antibody that detects HNK-1, a carbohydrate epitope frequently present on adhesion molecules (58), demonstrated that this epitope was present on p120 from brain but not from bursa or thymus, a result that agrees with the analysis of DM-GRASP (14, 60). p120 from DT40(T) and 30B3 did not contain the HNK-1 epitope (data not shown).

DNA sequences of a cDNA clone from a DT40(T) library and products of reverse transcription PCR (RT-PCR) using mRNA from v-*rel*-tumor cells were identical to DM-GRASP. A 0.7-kb *Pst*I-*Eco*RI fragment derived from the 3′-end region of a DM-GRASP cDNA (Fig. 3B) was used to screen a cDNA library prepared from DT40(T) mRNA. Restriction enzyme mapping of one positive clone identified a 1.8-kb insert with a 1.2-kb open reading frame and 0.6 kb of untranslated 3′-end region homologous to DM-GRASP. Sequence analysis of 308 nucleotides from the 5′ end of the insert (nucleotides 912 to 1220 [14]) and 196 nucleotides from the 3′-end region of the open reading frame (nucleotides 1671 to 1867) demonstrated that these two regions were identical to DM-GRASP. RT-PCR using mRNA from 30B3 or DT40(T) cells and combinations of N and C primers derived from the 5′-end, middle, and 3′-end regions of DM-GRASP (Fig. 3B) produced DNA fragments that appeared to be identical to fragments amplified in parallel from DM-GRASP DNA (data not shown). Sequence analysis of the PCR products from nucleotides 525 to 668 and from nucleotides 787 to 911 demonstrated that these regions were identical to DM-GRASP.

p120 is expressed from a 5.3-kb transcript. Northern analysis of poly(A)$^+$-selected RNA using entire DM-GRASP cDNA clone as a probe identified two RNA species: a 5.3-kb transcript and a 5-kb transcript. The 5.3-kb RNA was specifically present in DT40(T) and 30B3 cells (Fig. 3A, lanes 3 and 4) but not in DT40 or DT40(CSV) cells. An RNA of the same size was expressed in adult chicken brain, thymus, and bursa of Fabricius (lanes 6, 7, and 8, respectively). Very low levels of expression were seen in spleen (lane 10) but not in liver or bursal B cells (lanes 9 and 5, respectively). Overexposure of the filter did not show the 5.3-kb transcript in lanes 1, 5, and 9 (data not shown). Further, the 5.3-kb transcript was detected by the DM-GRASP probe in CEF and DT95 cells following transfection and infection by a retroviral vector that expresses v-*rel* or c-*rel* (data not shown). The 5-kb transcript, however, was widely expressed in a variety of cells and normal tissues (Fig. 3A).

To examine the relationship of the 5- and 5.3-kb RNAs, fragments derived from the 3′-end, middle, and 5′-end regions of the DM-GRASP cDNA clone were used as probes for Northern analysis. The 3′-end probe (Fig. 3B, *Pst*I-*Eco*RI fragment) and the middle probe (*Xba*I-*Pst*I fragment) hybridized only to the 5.3-kb RNA species. Surprisingly, the 5′-end probe of 249 nucleotides (*Eco*RI-*Xba*I fragment), which contained the amino acid sequence identified from purified p120, hybridized to both RNA species and reacted more strongly with the 5-kb RNA (data not shown). These data indicate that the 5-kb poly(A)$^+$ RNA contains sequences homologous to the 5′-end region of the DM-GRASP clone.

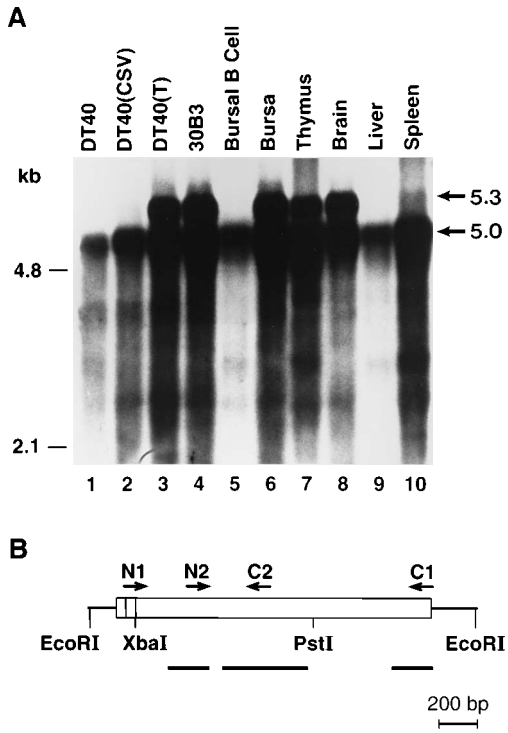

FIG. 3. (A) A 5.3-kb transcript is induced in v-*rel*-tumor cells and is expressed in the adult tissue of bursa, thymus, and brain. Poly(A)$^+$-selected RNA (5 μg) from DT40, DT40(CSV), DT40(T), 30B3, and bursal B cells and from tissues of bursa, thymus, brain, liver, and spleen was separated on a 0.8% formaldehyde-agarose gel. The RNA molecules were transferred to a nylon membrane and hybridized with entire DM-GRASP cDNA clone. The filters were exposed to X-ray film for 20 h. The sizes of the rRNAs are indicated on the left side of the panel, and the sizes of two RNAs hybridized with DM-GRASP clone are indicated on the right side of the panel. (B) Diagram of DM-GRASP cDNA clone. The open box represents the open reading frame of DM-GRASP. The fine lines flanking the box represent untranslated regions. The shaded portion indicates the N-terminal amino acid sequences that were determined from the purified p120. Arrows above the box represent primers used for cDNA synthesis, PCR amplification, and DNA sequencing. The nucleotide sequences that have been determined from our clone or from the PCR products are indicated by thick lines. Restriction enzyme recognition sites are indicated.

In summary, the presence of the 5.3-kb poly(A)$^+$ RNA correlates with the distribution of p120 in the tumor cell lines and normal tissues, and this species was induced when cells were induced to express p120. We conclude, therefore, that p120 is expressed from this transcript.

p120 is expressed on bursal epithelial cells but not on B cells. The normal tissue distribution of p120 was examined and compared to the distribution of DM-GRASP. Cells prepared from bursa, spleen, thymus, bone marrow, and blood were analyzed. Immunofluorescence microscopy detected p120 only on a small population of cells in bone marrow and in peripheral blood (Table 1). Flow cytometric analysis, however, detected a low level of p120 expression in the thymus and in a small population of cells in the spleen (Table 1). No p120-positive cells were detected from bursal cell suspensions. Examination of embryonic cell suspensions demonstrated that p120 expression could be detected as early as day 4 of embryogenesis while cells from embryonic bursa were negative for p120 (Table 1).

Immunohistochemistry was used to measure the distribution

TABLE 1. Tissue distribution of p120

Sample[a]	Result of staining[b]
Cell suspensions	
Embryo	
Day 2	—
Day 4	20%
Day 7	10%
Day 12	5%
Embryonic bursa	
Day 11	—
Day 15	—
Adult tissue	
Bursa	— (—)
Spleen	— (10%)
Thymus	— (50%)
Bone marrow	15% (65%)
Leukocyte	3% (10%)
Erythrocyte	— (−)
Tissue sections	
Bursa of Fabricius	
Epithelial cells	+
B cells	−
Spleen	±
Thymus	
Thymocytes	+
Epithelial cells	+
Gut (submucosa)	+
Liver	
Sinusoids	+
Hepatocytes	−
Cecal tonsils	
Epithelial cells	+
Lymphocytes	−
Brain	+
Heart	−
Muscle	−

[a] Cell suspensions were prepared from whole chicken embryos or from tissues of 4-week-old birds. Mononuclear cells were purified from tissues by centrifugation of the cell suspensions through a Ficoll gradient (36). Cells were stained for indirect immunofluorescence (35). Tissue sections were prepared from 4-week-old chickens and analyzed by horseradish peroxidase staining as previously described (36).

[b] For cell suspensions, the percentage of positive cells is presented for both microscopic and flow cytometric analyses (the latter is shown in parentheses). —, no staining. Representative results from one of three independent analyses are shown. For tissue sections, +, positive stain; −, negative stain; ±, weakly positive stain.

of p120 in tissue sections from 4-week-old chickens. Frozen sections from different tissues were incubated with HY78 and developed with horseradish peroxidase. p120-positive cells were observed in a variety of tissues, including liver, spleen, thymus, brain, gut, cecal tonsils, kidney, and pancreas (Table 1). In liver, kidney, and spleen, the adventitia within the walls of blood vessels were strongly stained. The sinusoids of liver were also stained intensely (Fig. 4A). In the gut and cecal tonsils, p120 was detected on the border of the lamina and submucosa, in the interior of the villus, and in island-like structures that resembled blood vessels (Fig. 4B). In the brain, p120-positive cells did not appear to be associated with specific structures (Fig. 4C). In the bursa of Fabricius, p120 was expressed at high levels on cells that divided the cortex and medullary compartments of the follicles and on cells that compose the reticular network within the medulla (Fig. 4D). In agreement with the immunofluorescence analysis, p120 does not appear to be present on bursal B cells. Staining of spleen was difficult to define because of the complexity of the tissue. It appeared, however, that lymphoid cells within the white pulp were negative for p120. Both thymic epithelium and thymocytes stained weakly (data not shown).

The tissue distribution of p120 is consistent with that of DM-GRASP (14, 19, 71). In summary, DM-GRASP is primarily located in three kinds of tissues: neurons, epithelium, and some hematopoietic tissues. Heterogeneity in the size of DM-GRASP from different tissues has been described (60). To see whether the pattern of p120 from these tissues is also heterogeneous, bursa, thymus, and brain were homogenized in RIPA buffer and analyzed by immunoprecipitation followed by Western transfer. The result illustrates that proteins of different apparent molecular weight are present in these tissues: a 95-kDa molecule from brain, a 130-kDa protein associated with a 110-kDa molecule from bursal epithelium, and a 120-kDa species from thymus that was the same size as p120 from v-*rel* tumor cells (Fig. 5). It has been suggested that the heterogeneity of p120 is related to variation in glycosylation (60).

Although the complete cDNA clone encoding p120 was not isolated from our DT40(T) library, the data presented above demonstrated that p120 is DM-GRASP.

DM-GRASP is induced by both v-*rel* and c-*rel* in CEF, B-cell lines, and spleen cells. The expression of DM-GRASP is not restricted to v-*rel* tumors; it is also expressed in RP9, a *myc*-transformed B-cell line, and in BM2, a *myb*-transformed myeloid cell line. To examine the specificity of induction of DM-GRASP, CEF were transfected with pCSV in combination with pREV-v-*rel* or pREV-c-*rel*, which express v-*rel* or chicken c-*rel*, respectively. The expression of Rel protein, CSV viral protein, and DM-GRASP was monitored by immunofluorescence microscopy. The results demonstrated that both v-*rel* and c-*rel* induced DM-GRASP in fibroblasts and that c-*rel* appeared to induce DM-GRASP more rapidly than v-*rel* (Fig. 6A). c-Rel was present exclusively in the cytoplasm from the time of its first detection (approximately 3 days after transfection). v-Rel, however, appeared first in the nucleus and then migrated to the cytoplasm beginning approximately 5 days after transfection. Using dual immunofluorescence staining for v-Rel and p120, most of the DM-GRASP positive cells were cells with the cytoplasmic v-Rel. The first observed induction of DM-GRASP correlated with the relocalization of v-Rel to the cytoplasm (data not shown). Based on data from several experiments, the ratio of DM-GRASP-positive cells to nuclear v-Rel cells was 1:12 while the ratio of DM-GRASP-positive cells to cytoplasmic v-Rel cells was 1.2:1 (cells were analyzed from day 4 to day 6 after transfection), although the possibility that low-level expression of nuclear v-Rel was not detected by microscopy cannot be excluded.

The induction of DM-GRASP was also examined in two B-cell lines. The DT95 cell line was cocultivated with CEF transfected with REV-v-*rel* or REV-c-*rel* and was examined for the expression of either v-*rel* or c-*rel* and DM-GRASP by immunofluorescence microscopy. Both v-*rel* and c-*rel* induced DM-GRASP in DT95 with comparable kinetics (Fig. 7A). Northern analysis of mRNA from DT95 and CEF showed that induction of DM-GRASP was coincident with the appearance of a 5.3-kb mRNA that reacted specifically with the DM-GRASP clone (data not shown). Similar results were obtained with infection of DT40 (data not shown). In addition, examination of 24 clones derived from spleen mononuclear cells infected in vitro by REV-v-*rel* or REV-c-*rel* demonstrated that both v-*rel* and c-*rel* induced expression of DM-GRASP in these clones, which included T cells, B cells, and non-T, non-B cells (presumably myeloid cells). Treatment of CEF and DT40 with phorbol 12-myristate 13-acetate also induced transient expression of DM-GRASP in these cells (data not shown).

Previous studies have shown that v-*rel* induces MHC class I,

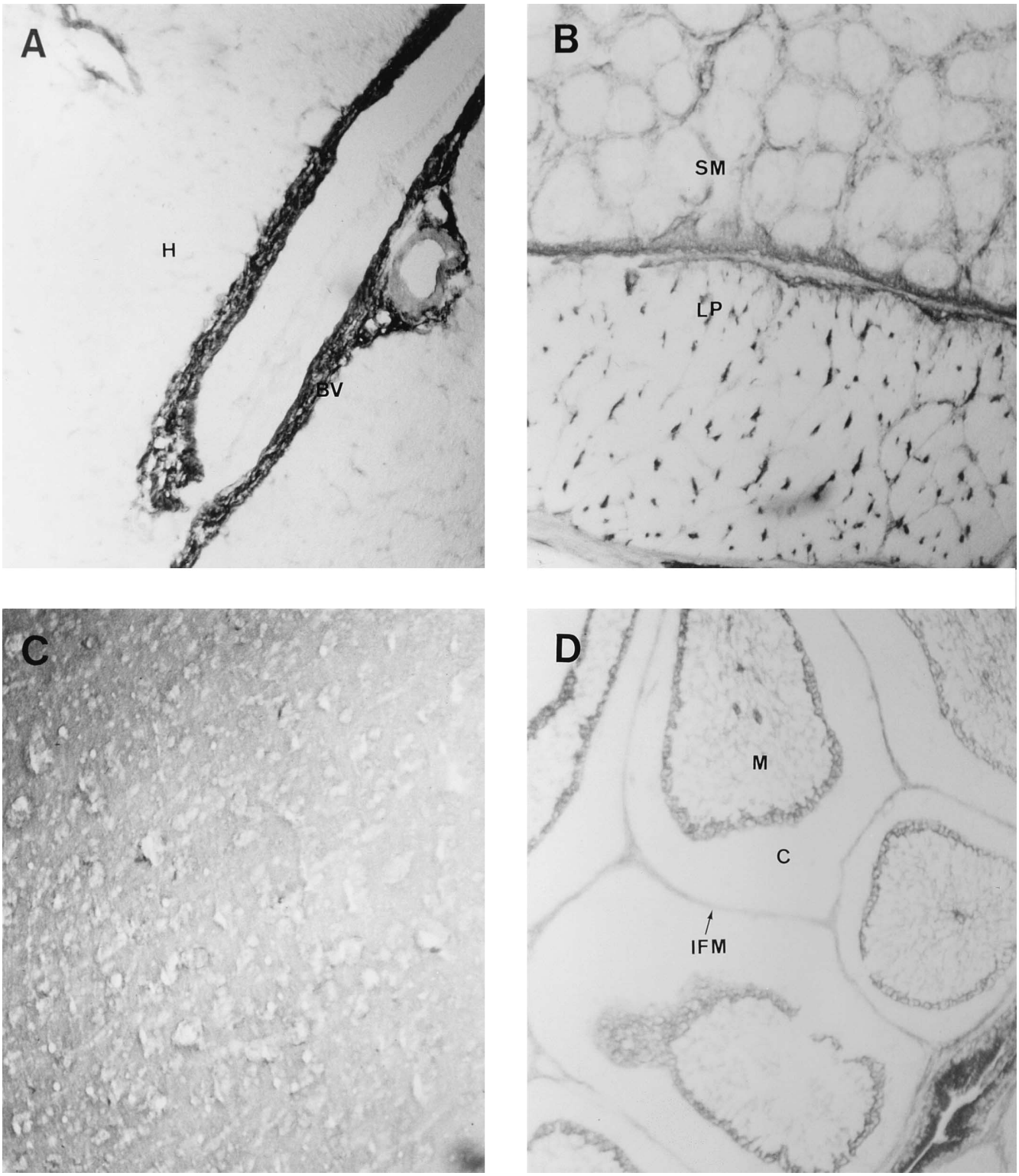

FIG. 4. p120 is expressed in a variety of chicken tissues but not in bursal B cells. p120 is associated with epithelial cells in the border that divides medulla and cortex and within the medulla of bursa of Fabricius but is not expressed on bursal B cells. Tissue sections from 4-week-old birds were stained with HY78 monoclonal antibody and developed with horseradish peroxidase. Positive cells appear deep brown. (A) Liver; (B) gut; (C) brain; (D) bursa of Fabricius. BV, blood vessel; C, cortex; H, hepatocyte; IFM, interfollicular membrane; LP, lamina propria; M, medulla; SM, submucosa.

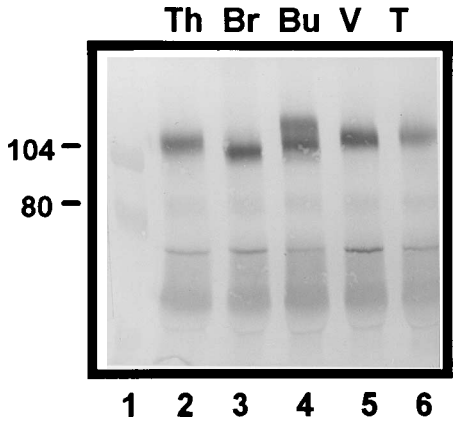

FIG. 5. p120s from neuronal, epithelial, and hematopoietic tissues are heterogeneous in size. Homogenized lysate from 10-mg tissues of thymus (Th), brain (Br), or bursa of Fabricius (Bu) or lysate from 10^7 cells of 30B3 (V) or DT40(T) (T) were incubated with HY78 monoclonal antibody and precipitated by protein A-Sepharose beads. The precipitates were separated by SDS–10% PAGE and transferred to a nitrocellulose membrane. The filter was stained with HY78 antibody and developed with streptavidin-alkaline phosphatase. Prestained marker proteins were loaded on lane 1, and their sizes (in kilodaltons) are indicated on the left side of the panel.

MHC class II, and IL-2R more efficiently than c-*rel* (35). A quantitative analysis was used to examine the induction of DM-GRASP and demonstrated that the ratio of DM-GRASP/v-Rel was approximately fivefold more than that of DM-GRASP/c-Rel in DT95 (Fig. 7B). In contrast, no significant difference of induction of DM-GRASP by v-*rel* and c-*rel* was observed in CEF (Fig. 6B).

HY78 monoclonal antibody inhibits the proliferation of v-*rel*-transformed cells. To determine whether DM-GRASP plays a role in v-*rel*-induced tumorigenesis, purified monoclonal antibody HY78 against DM-GRASP was added to v-*rel*-transformed cells proliferating in vitro. Transformed cells were suspended in culture medium with one of several different monoclonal antibodies, and the number of cells was monitored for approximately 2 weeks. When necessary, the cells were diluted into fresh medium to keep the cell density between 0.1 $\times 10^6$ and 2.0 $\times 10^6$/ml. The total number of cells produced by a culture was calculated on the basis of the dilution. The presence of 1 μg of HY78 in 1 ml of medium with 10^5 cells inhibited the proliferation of 30B3 cells, a v-*rel*-transformed B-cell line (Fig. 8). The cells grew as bigger aggregates than did control cells. We observed a similar inhibition of proliferation of both S2A3 and 27L2, two v-*rel*-transformed cell lines (data not shown). There was no effect of HY78 on the growth of DT40(CSV), a *myc*-transformed cell line. The monoclonal antibody, HY82, which recognizes a v-*rel*-induced surface protein (37), exhibited no effect on the growth of 30B3, S2A3, or 27L2. It is important to note that HY78 did not inhibit the growth of DT40(T), a cell line transformed by elevated expression of *myc* that also expresses DM-GRASP as a result of superinfection with REV-T(CSV) (Fig. 8). Similarly, HY78 also showed no inhibitory effect on the growth of BM2, a myeloid cell line transformed by v-*myb* that also expresses DM-GRASP (Fig. 8).

DISCUSSION

p120 is DM-GRASP, and its expression in v-*rel*-induced B-cell lymphomas is apparently ectopic. Using the N-terminal 27-amino-acid sequences obtained from immunopurified p120, we identified a gene, designated DM-GRASP, which encodes a protein whose N-terminal amino acid sequence is identical to that of p120. Results from immunological cross-reaction, mRNA analysis, cDNA cloning from a DT40(T) library, reverse PCR, and DNA sequencing of a cDNA clone and the PCR products provide conclusive evidence that p120 is DM-GRASP. Furthermore there is heterogeneity in the apparent

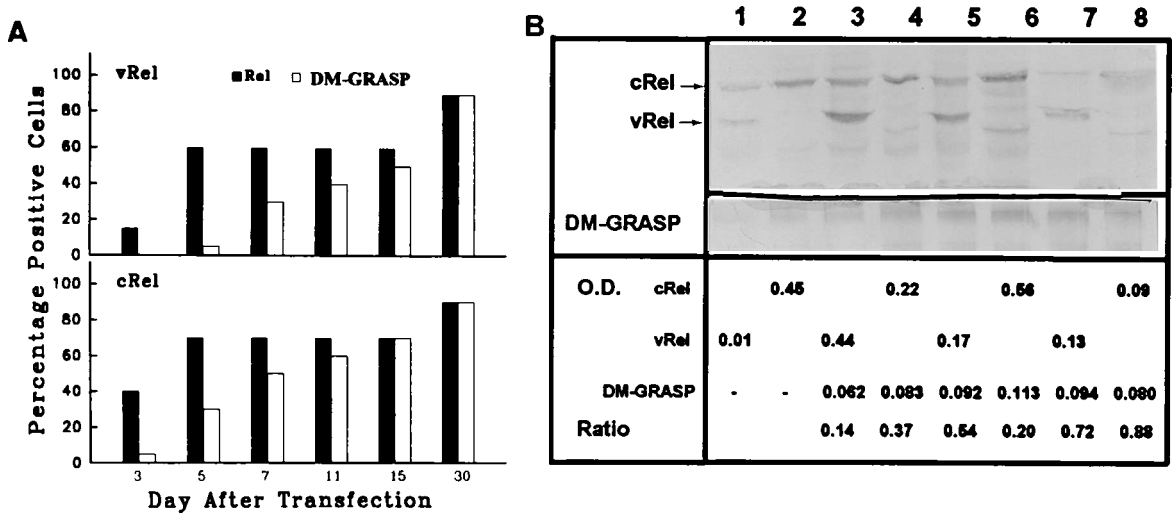

FIG. 6. Induction of DM-GRASP by v-*rel* and c-*rel* in CEF. (A) Kinetics of induction of DM-GRASP by Rel. CEF was transfected by pREV-v-*rel*(CSV) (top panel) or pREV-c-*rel*(CSV) (bottom panel), and cells were trypsinized and analyzed by immunofluorescence staining at the time indicated using anti-Rel or HY78 monoclonal antibodies. Positive cells were counted with a UV microscope and expressed as a percentage of the total number of cells counted. Solid bar, Rel; open bar, DM-GRASP. Representative data of one experiment (from a total of three independent experiments) are shown. (B) Quantitative analysis of Rel and DM-GRASP on CEFs transfected by pREV-v-*rel* or pREV-c-*rel*. CEF transfected with REV-v-*rel* (lanes 1, 3, 5, and 7) or REV-c-*rel* (lanes 2, 4, 6, and 8) were analyzed by Western analysis (Rel) or immunoprecipitation followed by Western analysis (DM-GRASP) using anti-Rel or HY78 antibodies. Cells (1×10^5 or 5×10^6) were used for the analysis of Rel or DM-GRASP, respectively, at day 3 (lanes 1 and 2), day 6 (lanes 3 and 4), day 9 (lanes 5 and 6), and day 12 (lanes 7 and 8) after transfection. The proteins were separated by SDS–10% PAGE and transferred to nitrocellulose membranes. The filters were incubated with the indicated antibodies and developed with streptavidin-alkaline phosphatase. The membranes were scanned by an XRS scanner, and the corresponding optical density readings are presented in the bottom panel. Ratio is calculated as DM-GRASP unit/Rel unit. Rel proteins and DM-GRASP are indicated by arrows.

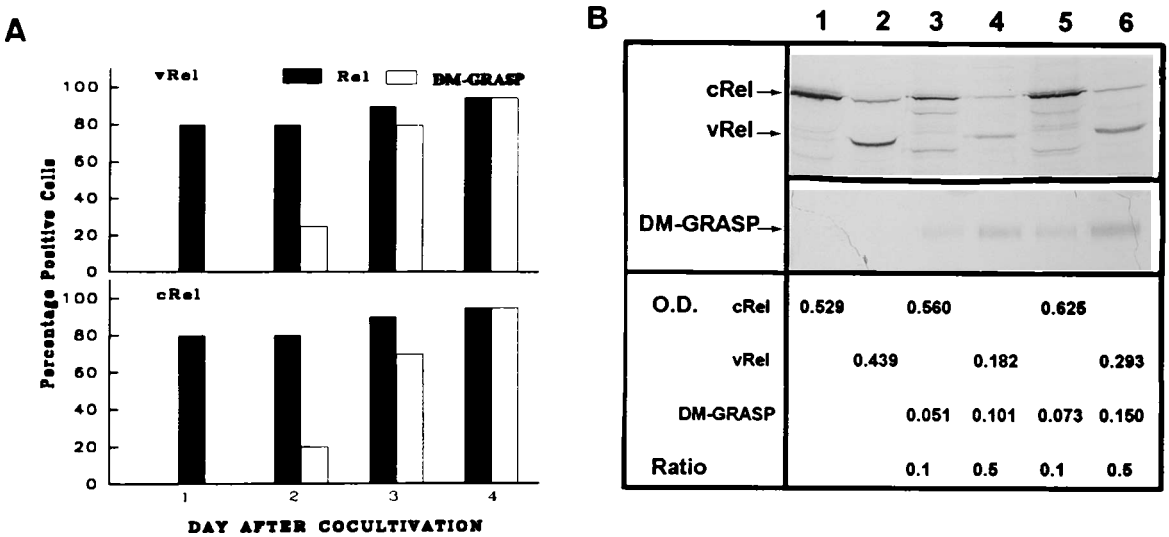

FIG. 7. Induction of DM-GRASP by v-*rel* and c-*rel* in DT95. (A) Kinetics of induction of DM-GRASP by Rel. DT95 cells were cocultivated with REV-v-*rel*- or REV-c-*rel*-transfected CEF for 1 h and collected for further culture. Cells were stained by immunofluorescence with anti-Rel or HY78 monoclonal antibodies at the times indicated and examined with a UV microscope. Solid bar, Rel; open bar, DM-GRASP. (B) Quantitative analysis of Rel and DM-GRASP proteins on DT95 cells expressing v-*rel* or c-*rel*. The methods used are described in the legend for Fig. 6. DT95/vRel (lanes 1, 3, and 5) or DT95/cRel (lanes 2, 4, and 6) were analyzed at day 2 (lanes 1 and 2), day 3 (lanes 3 and 4), and day 4 (lanes 5 and 6) after cocultivation. Rel proteins and DM-GRASP are indicated by arrows.

molecular weight of DM-GRASP (60) and the pattern of size distribution in the various tissues agrees with the pattern of size distribution of p120. On the basis of this information, we conclude that p120 is DM-GRASP.

Analysis of normal chicken tissues showed that p120 is not expressed in either embryonic or adult bursal hematopoietic cells, although it is expressed at high levels in bursal epithelial cells. It also appears not to be expressed in peripheral B cells. Our observation is supported by the studies of BEN (another name for DM-GRASP), which was identified by a monoclonal antibody generated by using bursal epithelial components as immunogen (59). Dual immunofluorescence analysis on tissue sections from embryonic and adult bursa demonstrated that the expression of DM-GRASP/BEN is restricted to the epithelial cells. Similar examination on tissue sections from cecal tonsils and the Harderian gland, which contain mature and activated B cells, showed that DM-GRASP/BEN-positive cells are immunoglobulin M negative (20). Although we and others do not have data to show whether DM-GRASP is expressed on pro- and/or pre-B cells because of the lack of suitable cellular markers, current observations support the conclusion that the induction of DM-GRASP by v-*rel* in B-cell lymphomas, at least in bursal B-lymphomas, is apparently ectopic.

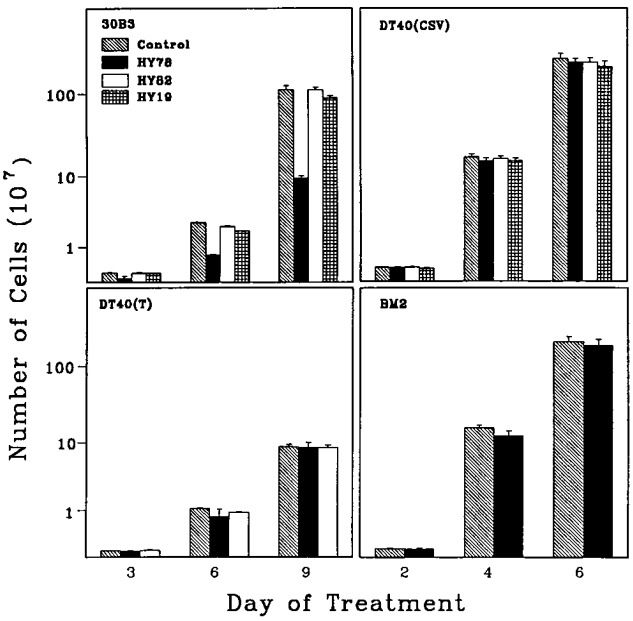

FIG. 8. HY78 monoclonal antibody inhibited the growth of v-*rel*-tumor cells in culture. Cell (10^5) were incubated in media containing immunopurified monoclonal antibodies for 2 or 3 days. The cells were then counted and resuspended at the same conditions used on day zero. The effect of 1 to 20 μg of antibodies on the growth of these cell lines was similar. Only the effect of 1 μg of antibodies on the growth of the cells is shown. Control, PBS; HY19, anti-immunoglobulin M; HY78, anti-DM-GRASP; HY82, anti-p75. Data of three independent experiments with 30B3, DT40(CSV), and BM2 are presented as means ± standard errors. Data of two independent experiments with DT40(T) are presented as means ± standard errors.

Studies of the expression of DM-GRASP/BEN in chicken hematopoietic cells showed that DM-GRASP/BEN is expressed in immature thymocytes (CD4/CD8 double positive cells) as well as in progenitor cells of myeloid and erythroid lineage. Expression is lost as progenitor cells proliferate and differentiate to mature cells as examined by an in vitro colony formation assay (19). Mature myeloid cells, such as macrophages, granulocytes, thrombocytes, and erythrocytes, do not express DM-GRASP/BEN (19). These observations suggested that DM-GRASP/BEN is a stage-specific rather than a lineage-specific differential antigen expressed by immature hematopoietic cells. v-*rel* is capable of transforming both immature and mature hematopoietic cells, including mature T cells and myeloid cells (6). All v-*rel*-transformed cells examined, including cell lines derived from tumors and clones obtained from in vitro infection, were DM-GRASP positive. These results indicate that the induction of DM-GRASP expression is tightly associated with the oncogenic process mediated by v-*rel*. Nevertheless, the pathway of induction of DM-GRASP by v-*rel* in B cells may be different from that in mature T cells and myeloid cells.

DM-GRASP is a novel adhesion molecule belonging to the immunoglobulin superfamily. The DM-GRASP cDNA was originally isolated from a chicken embryo cDNA library (14). It contains an open reading frame of 588 amino acids, which consists of a signal peptide, five immunoglobulin-like domains, a transmembrane region, and a short cytoplasmic tail. The DM-GRASP amino acid sequence was found to be homologous to several members of the immunoglobulin superfamily, including myelin-associated glycoprotein (43), neuron cell adhesion molecule (N-CAM) (9), and the carcinoembryonic antigen (10). The closest relative of DM-GRASP is a human metastatic melanoma marker, MUC18 (44), which has a similar structural arrangement. However, on the basis of the relatively low overall similarity (about 20%) and the fact that tissue distribution of these two proteins is different, it is unlikely that MUC18 is the human homolog of DM-GRASP. Therefore, DM-GRASP is a new member of the immunoglobulin superfamily.

Our Northern analysis using the DM-GRASP cDNA as a probe detected a 5.3-kb transcript from cell lines expressing v-*rel*. This transcript is not present in the cell lines transformed by *myc* that do not express DM-GRASP. The 5.3-kb mRNA was also detected in tissues that normally express DM-GRASP. However, the size of the mRNA we detected is larger than what has been previously reported (4- to 4.5-kb transcript) (60, 71). Our Northern analysis also showed that there was a 5-kb transcript that hybridized to the 5′-end regions of the DM-GRASP cDNA clone. The expression of the 5-kb transcript did not correlate with the expression of p120 either in tumor cells or in normal tissues (Fig. 3A). The 5-kb mRNA may be expressed from a different gene. It is also possible that this species is a product derived from differential splicing, although probes representing more than 75% in the DM-GRASP cDNA do not hybridize to this species. No consensus donor or acceptor sites were detected in the DM-GRASP cDNA clones, although there may be alternative splicing upstream of the sequenced region. The available DM-GRASP cDNA clones are significantly smaller than the 5.3-kb transcript. A more complete understanding of these phenomena will require a complete analysis of the organization of DM-GRASP gene and its transcripts.

DM-GRASP is involved in intercellular adhesion and may promote the growth of v-*rel* tumors. Members of the immunoglobulin superfamily have diverse functions. For instance, N-CAM plays a role in neuronal tissue formation and growth (21). ICAM-1 is involved in the interaction of T and B cells (63) and is essential for cell-mediated cytotoxicity (65). VCAM-1 participates in the recruitment of cells from the bloodstream to sites of inflammation (57). All these functions require recognition and binding activity that apparently rely on the immunoglobulin-like structures. It has been suggested that DM-GRASP functions in axonal and lateral motor column formation (14, 59, 72). In addition, DM-GRASP has been implicated as a hematopoietic differentiation-stage marker and may be involved in T-cell activation (19, 20). The distribution pattern of DM-GRASP in the bursa of Fabricius is quite interesting, as it is expressed at high levels in the follicle epithelium which also forms reticular networks within the medulla (Fig. 4D). It has been suggested that bursal epithelial cells (which include stromal and dendritic cells) play an important role in B-cell development. The distribution pattern of DM-GRASP in the bursa of Fabricius (on the cells that interact with B cells) and the subcellular localization of DM-GRASP (on the surface membrane) suggest a possible involvement of DM-GRASP in this process; DM-GRASP could serve as a ligand or receptor during the contact between an epithelial cell and a B cell.

We and others observed that DT40(T) and v-*rel*-transformed cells grew in large aggregates (25), and this aggregation is apparently required for the growth of the tumor cells (27a, 76). DM-GRASP could play a role in the cell aggregation, acting in a homophilic fashion when expressed on a cell line derived from human embryonic kidney (71). However, there is as yet no evidence to demonstrate that DM-GRASP is directly involved in the aggregation of v-*rel* tumor cells. In contrast, we observed that BM2 and RP9, both of which express DM-GRASP, proliferate as single cells or as small aggregates (3 to 10 cells in aggregation compared with 50 to 200 cells for v-*rel*-transformed cells). It is possible that DM-GRASP plays different roles in different tissues or cells. The finding that DM-GRASP from brain, but not from bursa or v-*rel* tumor cells, contains the HNK-1 antigen suggests a diversity in the potential adhesive activity of this protein.

Assays that employed specific antibodies indicated that DM-GRASP may play a role in the neurite extension (14), and it was involved in intercellular adhesion (71). We had found that the HY78 monoclonal antibody inhibited the growth of v-*rel* tumor cells in culture (Fig. 8). However, HY78 did not block the aggregation of either 30B3 or DT40(T) cells. In contrast, these cells grew in larger aggregates. It seems unlikely that the inhibition of cell growth was the result of the formation of large aggregates since no inhibition was seen on the growth of DT40(T) cells. The observation that HY78 did not inhibit the growth of DT40(T) cells indicates that DM-GRASP may play a specific role in v-*rel*-transformed cells, since DT40(T) cells are not transformed by v-*rel* but by *myc*. The similar mechanism may apply for BM2, whose growth was not interfered with by HY78. BM2 is a myeloid cell line transformed by *myb* and expresses DM-GRASP. Since DM-GRASP is normally expressed in myeloid cells, this protein may not play a role in the *myb*-mediated transformation in this cell. The antibody-induced inhibition of cell growth is not mediated by complement, since the serum used in the media has been heat inactivated. These data demonstrated the specificity of the inhibitory effect of the antibody for v-*rel* tumors.

Experiments using NF-κB antisense oligonucleotides, which targeted the expression of the p65 subunit, demonstrated inhibition of tumor growth accompanied by the loss of cell adhesion to an extracellular matrix (33, 53). NF-κB antisense oligonucleotides also inhibited the induction of cell adhesion by phorbol ester (23). These data suggested that cell adhesion, which is mediated, at least in part, by the activity of NF-κB, is an important event in tumor development. It will be important to see whether the NF-κB antisense oligonucleotides block the transformation by v-*rel* and how this affects the expression of DM-GRASP. The results from such experiments should provide more information on the significance of the induction of DM-GRASP by v-*rel* in the tumor cells.

What is the mechanism of induction of p120 by v-*rel*? In addition to the expression of DM-GRASP in v-*rel* tumor cells, overexpression of v-*rel* and c-*rel* can induce DM-GRASP expression in CEF, B-cell lines (DT40 and DT95), and spleen mononuclear cells. Both v-Rel and c-Rel were apparently in the cytoplasm when they induced DM-GRASP expression in CEF cells. These observations suggest that v-Rel and c-Rel may use a similar mechanism to induce DM-GRASP. One possibility is that Rel interacts directly with the regulatory region of the DM-GRASP gene; another possibility is that Rel induces a cellular factor that is responsible for the induction of DM-GRASP. Although we cannot exclude such mechanisms, they do require Rel to be in the nucleus and our data suggest

that there is a correlation between the expression of DM-GRASP and cytoplasmic localization of v-Rel. Alternatively, induction of DM-GRASP could be mediated by other members of the NF-κB family. In this case, the overexpression of Rel in the cytoplasm would sequester IκBs, resulting in the release of NF-κB into the nucleus. The induction of DM-GRASP by phorbol 12-myristate 13-acetate, which is an activator of NF-κB, supports this model. In this model, the expression of DM-GRASP would normally be regulated by members of the NF-κB family.

Several adhesion molecule genes that contain NF-κB binding sites in their regulatory regions have been identified, including ICAM-1 (73), VCAM-1 (69), and endothelial leukocyte adhesion molecule 1 (ELAM-1) (75). Both ICAM-1 and VCAM-1 belong to the immunoglobulin superfamily; ELAM-1 is a member of the selection family. Another molecule, CD11b, which is a member of the integrin family, also appears to be regulated by NF-κB (23). It remains to be determined whether there are κB sites in the DM-GRASP gene. Cloning and analysis of the 5′-end regulatory region of this gene should clarify this issue.

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