Characterization of the gag/Fusion Protein Encoded by the Defective Duplan Retrovirus Inducing Murine Acquired Immunodeficiency Syndrome

MING HUANG\(^1\) AND PAUL JOLICOEUR\(^{1,2*}\)

Laboratory of Molecular Biology, Clinical Research Institute of Montreal, Montreal, Quebec H2W 1R7,\(^1\) and Département de Microbiologie et d’Immunologie, Université de Montréal, Montréal, Quebec H3C 3J7,\(^2\) Canada

Received 21 May 1990/Accepted 11 September 1990

Murine acquired immunodeficiency syndrome is induced by a defective retrovirus. Sequencing of this defective viral genome revealed a long open reading frame which encodes a putative gag/fusion protein, N-MA-p12-CA-NC-COOH, (D. C. Aziz, Z. Hanna, and P. Jolicoeur, Nature (London) 338:505–508, 1989). We raised a specific antibody to the unique p12 domain of this gag fusion precursor, Pr60\(^{505}\). We found that Pr60\(^{505}\) was indeed encoded by the defective viral genome both in cell-free translation reticulocyte extracts and in infected mouse fibroblasts. Pr60\(^{505}\) was found to be myristylated, phosphorylated, and attached to the cell membrane, like other helper murine leukemia virus (MuLV) gag precursors. Pr60\(^{505}\) was not substantially cleaved within the nonproducer cells and was not released from these cells. However, in the presence of helper MuLV proteins, it formed phenotypically mixed particles. In these particles, Pr60\(^{505}\) was only partially cleaved. In helper MuLV-producing cells harboring the defective virus, a gag-related p40 intermediate was generated both intracellularly and extracellularly. In these cells, Pr60\(^{505}\) appeared to behave as a dominant negative interferer, interfering with proper cleavage of helper Pr66\(^{505}\). Our data indicate that Pr60\(^{505}\) is a major (and possibly the only) gene product of the defective murine acquired immunodeficiency syndrome virus and is likely to harbor some determinants of pathogenicity of this virus.

The Duplan strain of murine leukemia virus (MuLV) was initially isolated from organs of X-irradiated C57BL/6 mice and subsequently passaged several times in these mice as a crude extract (9, 25, 27, 30). This crude virus stock induced a severe immunodeficiency syndrome (25, 31, 32) which has been designated murine acquired immunodeficiency syndrome (32). Among the several MuLVs present in this crude virus extract, the pathogenic agent has been identified by us (1) and others (4) as a 4.8-kbp defective MuLV. The presence of this defective virus in mice appears to be sufficient for initiation and progression of the immunodeficiency syndrome, since helper-free stocks of the defective virus were found to be highly pathogenic (17). Interestingly, we found that this defective virus induced oligoclonal expansion of target cells and therefore behaves as an oncogenic virus (17).

Sequencing of this defective viral genome revealed major deletions in pol and env with a relatively well-conserved gag region (1). A long open reading frame corresponding to the gag/fusion precursor with several modifications in the gag p12 region was identified. Because the defective viral genome appears to harbor sufficient sequences to induce disease, the identification and characterization of the proteins encoded by this genome represent an important step in understanding the exact molecular mechanism of its pathogenicity.

In the present work, we studied the putative gag/fusion protein, since it represents a likely candidate viral gene product which may be involved in the disease process. We found that this gag/fusion protein was indeed encoded by the defective viral genome both in cell-free translation reticulocyte extracts and in fibroblasts harboring the defective provirus, thus representing a major viral protein. Processing, posttranslational modifications, intracellular localization, and assembly of this protein were studied. In addition, a polyclonal antibody which recognizes this protein specifically was produced.

MATERIALS AND METHODS

Reagents, radioisotopes, and antisera. Rabbit reticulocyte lysates was purchased from Promega Biotech. [9,10\(^{3}\)H]myristic acid (53 Ci/mmol), \(^{32}\text{P}\) (0.8 mCi/ml); and \([\text{S}^{35}\text{S}]\)methionine (>1.000 Ci/mmoll) were purchased from Amersham Corp. Goat anti-MuLV gag antibodies were obtained from Program and Logistics, Viral Oncology, National Cancer Institute, Bethesda, Md. Each antiserum was checked by immunoprecipitation and found to react specifically with Pr65\(^{505}\) and with individual gag proteins labeled with \([\text{S}^{35}\text{S}]\)methionine (p30 and p12) (see below) or \([\text{H}]\)leucine (p15 and p10) (data not shown).

Protein synthesis in vitro. The cloned 4.2-kbp KpnI-cleaved Duplan genome (Du5H) (nucleotides 417 to 4648 in reference 1) was inserted into the KpnI site of a GEM-3 vector. The plasmid was cut by restriction enzymes SacI and/or EcoRI, and linearization of the DNA was checked on a 1% agarose gel. Linearized DNA (5 μg) was used for transcription in vitro with SP6 RNA polymerase, as previously described (29), in 50X reaction buffer (300 mM NaCl, 10 mM Tris hydrochloride, 5 mM EDTA) containing ATP, CTP, UTP, and GTP, each at 500 μM; 10 mM dithiothreitol; 80 to 100 U of RNase inhibitor (RNasin; Promega); and 25 μl of SP6 RNA polymerase. The synthesized 4.2-kb RNA species was checked by formaldehyde gel and used for rabbit reticulocyte translations as recommended by the manufacturer, with 50 μCi of \([\text{S}^{35}\text{S}]\)methionine. Translation reaction was performed for 15, 30, 45, or 60 min at 30°C with 35 μl of nuclelease-treated rabbit reticulocyte lysate (Promega)–1 μl of 1 mM methionine-free amino acid mixture (Promega)–1 μg of...

\(*\) Corresponding author.
synthesized RNA in 2 μl of H2O-5 μl of [35S]methionine (1,200 Ci/mmol) at 10 mM Ci/ml-1.25 μl of RNasin (Promega) RNase inhibitor at 40 U/μl-5.75 μl of H2O in a total volume of 50 μl. The resulting products were inspected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with or without immunoprecipitation with an appropriate antibody. The pattern was found to be the same for different time points, and therefore, to synthesize higher levels of proteins, we routinely used a 45-min incubation time.

Labeling of cells and immunoprecipitation. SIM.R mouse fibroblasts (39), SIM.R infected with GtT, MuLV (39) and/or transfected with defective Du5HNeo DNA (1), the Ψ2 packaging cell line producing Moloney MuLV particles (28), and the Ψ2 cell clone transfected with defective Du5HNeo DNA (17) have all been described previously. Cells (106) seeded in 60-mm-diameter Petri dishes were labeled for 3 h with [35S]methionine (100 μCi/ml) or [3H]myristic acid (500 μCi/ml) in methionine-free or complete Dulbecco modified Eagle medium, respectively. Labeling with 32PP (0.7 mM/ml) was performed in 1.5 ml of Dulbecco modified Eagle medium lacking phosphate and containing 10% dialyzed serum in 4-h starvation period and a 5-h labeling period were used. Immunoprecipitation was done essentially as previously described (16), except for 32P labeling; cold Tris-buffered saline was used to wash the cells instead of phosphate-buffered saline; and 2 mM EDTA was included in the lysis buffer to prevent phosphorylation subsequent to cell lysis (44). Briefly, cells were lysed in a lysis buffer containing 0.01 M Tris hydrochloride (pH 7.4), 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride and clarified by centrifugation at 25 K for 20 min. Cell lysates were usually precleared by incubation with normal serum and Pansorbin (Calbiochem Behring) overnight on ice. The bacteria and immune complexes were removed by centrifugation. The supernatants were then incubated with appropriate antiserum (2 h) and Pansorbin (2 h) on ice. After being washed three times with lysis buffer, the immune complex and Pansorbin were suspended in sample buffer (2% SDS, 0.1 M Tris hydrochloride [pH 6.8], 2 mM EDTA, 5% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue), boiled for 3 min, and centrifuged to remove particles, and the supernatants were loaded on constant or 5 to 20% gradient SDS-PAGE gels. The gels were impregnated with 1 M sodium salicylate solution for 30 min, if necessary, before fluorography.

Cell fractionation. Fractionation was done essentially as already described (38). Briefly, labeled cells were suspended in hypotonic buffer (5 mM KCl, 1 mM MgCl2, 20 mM HEPES [N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] [pH 7.1]), incubated at 4°C for 10 min, broken by homogenization and passage through a 25-gauge needle, and then inspected under a phase-contrast microscope. The lysate was centrifuged at 1,000 × g for 5 to 10 min. The pellet was suspended in phosphate-buffered saline and further purified by passage through a sucrose cushion by the method of Gorski et al. (12). The resulting pellet was suspended in immunoprecipitation lysis buffer (16) and designated the nuclear fraction. After low-speed (1,000 × g) centrifugation, the supernatant was centrifuged again at 100,000 × g for 60 min at 4°C. The resulting supernatant consisted of the soluble portion of the cytoplasm, while the pellet designated the particulate fraction contained both intracellular and plasma membranes (3).

Virus labeling and purification. Infected SIM.R fibroblasts in 10-cm-diameter dishes were labeled with [35S]methionine as described above. Culture medium (7 to 8 ml) was collected and mixed with ~15 ml of unlabeled medium from Moloney MuLV-infected fibroblasts. Virions were pelleted by centrifugation at 150,000 × g for 60 min at 4°C, suspended in 0.5 to 0.8 ml of 10 mM Tris hydrochloride (pH 7.4), and layered onto a 10-ml 25 to 45% linear sucrose gradient. After centrifugation at 30,000 rpm in a Beckman SW41 rotor for 16 h, samples (0.5 ml per fraction) were collected from the bottom of the centrifuge tube. Sample of each fraction was used to check density and reverse transcriptase activity (21). A few fractions showing proper density and the peak of reverse transcriptase activities were pooled. Virions from these pooled fractions were pelleted again and used for immunoprecipitation.

Immunization and preparation of antibodies. Antibodies were raised as already described (13). A synthetic peptide (N-Asn-Leu-Pro-Pro-Leu-Ser-Lys-Gly-Pro-Val-Lys-Lys-Arg-COOH) made by I.A.F. Biochem International Institute (Montreal, Quebec, Canada) on the basis of nucleotides 1447 to 1485 in the Du5H sequence (1) was conjugated to thyroglobulin by a carbodiimide method. Briefly, 5 mg of the synthetic peptide in 0.5 ml of distilled water (pH 5.5) was constantly mixed with 25 mg of thyroglobulin (0.5 ml) and 115 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.5 ml) overnight at 4°C. Removal of excess coupling agent was accomplished by 24-h dialysis against 0.9% NaCl. The peptide (100 μg) conjugated with thyroglobulin was then emulsified in 1 ml of complete Freund adjuvant plus 1 ml of 0.9% NaCl and injected intradermally into each rabbit. The animals received booster injections of 50 to 100 μg of antigen every 4 to 6 weeks, and blood samples obtained 2 weeks after booster doses were tested for the presence of antibodies and used for immunoprecipitation experiments. Sera were diluted 50- to 100-fold for immunoprecipitation studies.

RESULTS

The defective Duplan viral genome encodes a putative gag/fusion protein. Previous sequencing of the defective Duplan viral genome (clone Du5H) revealed that large parts of pol and env had been deleted, with only small regions of these genes remaining, and that the gag region had been conserved (1). This gag gene showed a long open reading frame which encodes a putative complete gag/fusion protein with a stop codon located immediately after the gag p10 (NC) region. This putative gag precursor protein encoded N-p15 (MA)-p12-p30 (CA)-p10 (NC)-COOH. The p15, p30, and p10 protein regions showed high identity (80 to 95%) to homologous proteins of other MuLVs; however, the p12 amino acid sequence was shorter by 8 amino acid residues and showed significantly less identity (~50%) than p12 sequences from other MuLVs.

Synthesis of Pr60pol in vitro by reticulocyte extracts. To determine whether this defective viral genome had the ability to encode this putative gag/fusion protein and to find out whether other proteins were encoded by this genome, we first used the reticulocyte protein-synthesizing system programmed by defective viral mRNA. Du5H viral RNA encompassing the complete genome was inserted into a GEM-3 vector at the KpnI site downstream of the SP6 promoter. The resultant plasmid was linearized at the SacI or EcoRI site downstream of the inserted genome (Fig. 1A). In vitro transcription with SP6 polymerase yielded a unique RNA species of about 4.2 kb (data not shown). Translation of this
Presence of Duplan virus Pr60\textsuperscript{eag} in transfected fibroblasts.

To determine whether the Duplan virus encoded this Pr60\textsuperscript{eag} protein in vivo, a clone of SIM.R mouse fibroblasts transfected with defective Du5H DNA was isolated (nonproducer) (1). By Northern (RNA) blot analysis with Du5H DNA as the probe, we found that this clone produced only the expected 4.2-kb genome-size RNA but no other species (data not shown). These cells were also reinfected with a nonpathogenic ecotropic MuLV (G\textsubscript{t}T\textsubscript{2}) (39). Lysates from both cell cultures were analyzed by immunoprecipitation with various anti-gag sera after labeling with \[^{35}\text{S}\text{]}\text{methionine.}\n
Defective Du5H Pr60\textsuperscript{eag} was detected with anti-gag p15, p30, and p10, but not with anti-p12, in both nonproducer and MuLV-reinfected producer fibroblasts (Fig. 2). In non producer cells, no other proteins encoded by the Du5H genome were identified, suggesting that no larger gag-X precursor to Pr60\textsuperscript{eag} was synthesized and that Pr60\textsuperscript{eag} was not cleaved in these cells.

In MuLV-infected producer cells containing or not containing Du5H DNA, the expected additional proteins encoded by the helper MuLV were detected with some of these anti-gag sera, namely, the gag-pol Pr180 precursor, Pr65\textsuperscript{eag}, and cleaved p30 (Fig. 2). The cleaved p15 and p10 proteins were not expected to be detected in this experiment, as they lack methionine residues. The helper p12 protein was not detected in this experiment, most likely because of its low abundance. Interestingly, in the lysate from cells harboring both the defective viral genome and ecotropic G\textsubscript{t}T\textsubscript{2} helper MuLV, we observed an additional protein (p40) (Fig. 2, lanes 12 and 16) which was not present in lysates from cells infected with the helper alone. This protein reacted very strongly with anti-p30 and anti-p10 sera but apparently not with anti-p15 and anti-p12 sera. The faint band seen with anti-p15 serum seems to be an unspecific cross-reaction. The same pattern was also observed in cells harboring Du5H and reinfected with Moloney (Fig. 3C, lane 5; see Fig. 6) or ecotropic G\textsubscript{t}T\textsubscript{2} (Fig. 3C, lane 3) MuLV. Most likely, this p40 protein originates from helper Pr65\textsuperscript{eag}, whose cleavage may be partially impaired by the presence of mutated Pr60\textsuperscript{eag} (see Discussion).

Antibodies specific for Duplan virus gag p12. To be able to detect the defective gag/fusion protein specifically, we raised antibodies to its most unique region, p12. Our sequencing data revealed that the carboxy-terminal region of Duplan virus (Du5H) gag p12 had only 40 to 50% identity with the gag p12 proteins of other MuLVs and was not identical to any other nonviral proteins (1). A synthetic 13-amino-acid residue peptide was used for immunization of rabbits as described in Materials and Methods. Four of five rabbits immunized with this antigen produced detectable specific antibodies with reasonable titers, as they could immunoprecipitate Du5H Pr60\textsuperscript{eag} made in reticulocyte extracts or fibroblasts at dilutions of 50- to 100-fold (Fig. 3A and C). The immunoprecipitation was specific, as it was eliminated completely by preincubation of sera with 50 \(\mu\)g of a pure peptide (Fig. 3B, lanes 2 and 5), while an unrelated peptide had no effect (Fig. 3B, lanes 3, 6, and 7). However, in cell lysates, preincubation with the p12-related peptide eliminated other cellular proteins and the general background of the gel was lighter (Fig. 3B, lane 5). This could reflect the fact that these proteins share some epitopes with gag p12 or, more likely, that the peptide binds unspecifically to other proteins. While recognizing Du5H Pr60\textsuperscript{eag}, these antisera (R) did not immunoprecipitate the gag proteins of other strains of MuLVs, namely, neurotropic, ecotropic Cas-Br-E (pNE-8) MuLV (20), ecotropic B-tropic G\textsubscript{t}T\textsubscript{2}.
Myristylation of Duplan virus Pr60⁰⁰⁰⁰. Myristylation appears to be a common feature of gag polyproteins of many mammalian retroviruses (45). It is believed to be responsible for the membrane localization (14, 43) and processing (40, 43) of the MuLV gag precursor and for virus assembly and MuLV particle formation (23, 40, 43). Defective Duplan virus Pr60⁰⁰⁰⁰ has the same structure as myristylated Pr65⁰⁰⁰⁰ of helper MuLVs and has also conserved the second N-terminal amino acid residue, glycine (1), which is essential for attachment of the myristylated group (14, 42, 45).

To determine whether Pr60⁰⁰⁰⁰ was myristylated, the packaging cell line Ψ2 (28) was transfected with Du5H neo DNA (17). A G-418-resistant clone was selected and labeled with [³H]myristic acid for 3 h, and the cell lysate was analyzed by immunoprecipitation with anti-gag p30 serum. As expected, Pr65⁰⁰⁰⁰ from the helper Moloney MuLV was the only major myristylated protein detected in Ψ2 cells (Fig. 4, lane 1). In Ψ2 cells harboring the defective Du5H viral genome, an additional myristylated protein with an apparent molecular mass of 60 kDa was detected (Fig. 4, lane 2), indicating that defective Duplan Pr60⁰⁰⁰⁰ is myristylated.

Phosphorylation of Duplan virus Pr60⁰⁰⁰⁰. The gag precursor of MuLV has been shown to be phosphorylated, and p12 is the only phosphorylated protein in MuLV virions (8). Phosphorylation of p12 has been shown to be on serine residues. Our sequence of Du5H shows that it has the potential to encode a p12 protein with eight serine residues (1).

To determine whether Pr60⁰⁰⁰⁰ was phosphorylated, the Ψ2 cells used for the myristylation study were incubated with [³²P]P i for 5 h and analyzed by immunoprecipitation with anti-p30 serum. Helper Moloney Pr65⁰⁰⁰⁰ was found to represent a major species of phosphorylated proteins in Ψ2 cells (Fig. 5, lane 1). In Ψ2 harboring the defective Du5H viral genome, an additional major phosphorylated protein with an apparent molecular mass of 60 kDa was detected with both anti-p30 serum and specific R antiserum (Fig. 5, lanes 2 and 4), indicating that the defective Duplan Pr60⁰⁰⁰⁰ was phosphorylated.

Subcellular localization of Duplan virus Pr60⁰⁰⁰⁰. The myristylated Pr65⁰⁰⁰⁰ protein of MuLV is usually found attached to the plasma membrane (14, 43). To determine whether the Pr60⁰⁰⁰⁰ protein of the defective Duplan virus was also located in the plasma membrane, the clone of Ψ2 packaging cells used previously and harboring defective Du5H DNA was labeled with [³²P]methionine for 3 h and fractionated by differential centrifugation into cytosol, particulate (membrane), and nuclear fractions as described in Materials and Methods.

The viral proteins were then detected with anti-p30 serum. Most of the labeled proteins present in these cells and immunoprecipitated were in the membrane fraction, including the Pr65⁰⁰⁰⁰ and Pr60⁰⁰⁰⁰ proteins, respectively, from helper Moloney and defective Duplan viruses (Fig. 6). Very little of the gag precursors was found in other fractions. Two major cleavage products (p30 and p40), most likely originating from the helper Moloney Pr65⁰⁰⁰⁰ precursor (see Discussion), were present in these cells (Fig. 6, lanes 2 and 4). These were also located predominantly in the membrane fraction, as expected, since cleavage and processing of the gag/fusion protein are temporally and physically linked with the budding and formation of virions particles, both occurring at the plasma membrane (43, 49). Therefore, the Duplan
were extracts defective antiserum 1 (lanes 1 and 3), goat anti-gag p30 serum (lane 2), or normal rabbit serum (lane 7). A 12% constant SDS-PAGE gel was used. (B) Specificity of R antiserum immunoprecipitation. 35S-labeled proteins synthesized with DuSH RNA in reticulocyte extracts (lanes 1 to 3) (12% constant SDS-PAGE gel) or from \( \Psi^2 \) cells harboring the defective provirus (lanes 4 to 7) (5 to 20% gradient gel) were immunoprecipitated with (i) R2 antiserum (lanes 1 and 4), (ii) R2 antiserum preincubated with either a p12-related peptide (50 \( \mu \)g) (lanes 2 and 5) or the same amount of an unrelated peptide, namely, a DuSH peptide in the wrong open reading frame (lanes 3 and 6), or an antinatriuretic factor (lane 7). (C) Specificity of R2 antiserum for DuSH Pr60\(^{agg} \) (5 to 20% gradient gel). 35S-labeled proteins from mouse fibroblasts infected with ecotropic, neurotropic Cas-Br-E (pNE-8) MuLV (20) (lanes 1 and 2), with ecotropic, B-tropic G\(_T\) radiation leukemia virus (39) and DuSH defective MuLV (lanes 3 and 4), with ecotropic Moloney MuLV and DuSH defective MuLV (17) (lanes 5 and 6), or with amphotropic 4070-A MuLV (7) (lanes 7 and 8) were immunoprecipitated with either goat anti-gag p30 serum (lanes 1, 3, 5, and 7) or rabbit R2 antiserum (lanes 2, 4, 6, and 8).

FIG. 3. Characteristics of R antisera to defective DuSH Pr60\(^{agg} \) proteins. (A) Proteins synthesized with DuSH RNA by reticulocyte extracts were immunoprecipitated with sera (R1 to R5) from five different rabbits immunized with the synthetic p12-related peptide (lanes 1 and 3 to 6), goat anti-gag p30 serum (lane 2), or normal rabbit serum (lane 7). A 12% constant SDS-PAGE gel was used. (B) Specificity of R antiserum immunoprecipitation. 35S-labeled proteins synthesized with DuSH RNA in reticulocyte extracts (lanes 1 to 3) (12% constant SDS-PAGE gel) or from \( \Psi^2 \) cells harboring the defective provirus (lanes 4 to 7) (5 to 20% gradient gel) were immunoprecipitated with (i) R2 antiserum (lanes 1 and 4), (ii) R2 antiserum preincubated with either a p12-related peptide (50 \( \mu \)g) (lanes 2 and 5) or the same amount of an unrelated peptide, namely, a DuSH peptide in the wrong open reading frame (lanes 3 and 6), or an antinatriuretic factor (lane 7). (C) Specificity of R2 antiserum for DuSH Pr60\(^{agg} \) (5 to 20% gradient gel). 35S-labeled proteins from mouse fibroblasts infected with ecotropic, neurotropic Cas-Br-E (pNE-8) MuLV (20) (lanes 1 and 2), with ecotropic, B-tropic G\(_T\) radiation leukemia virus (39) and DuSH defective MuLV (lanes 3 and 4), with ecotropic Moloney MuLV and DuSH defective MuLV (17) (lanes 5 and 6), or with amphotropic 4070-A MuLV (7) (lanes 7 and 8) were immunoprecipitated with either goat anti-gag p30 serum (lanes 1, 3, 5, and 7) or rabbit R2 antiserum (lanes 2, 4, 6, and 8).

Assembly of Duplan viral Pr60\(^{agg} \) into virions. It has been shown before that attachment of MuLV Pr60\(^{agg} \) to the plasma membrane is essential for virus assembly (40, 43). However, cleavage of this gag precursor seems to occur only during budding or in virions (5, 8, 24, 43). Since Duplan virus Pr60\(^{agg} \) is myristylated and attached to the plasma membrane, it would be expected to bud and be incorporated into virions by itself, since the gag precursor appears to be sufficient for particle formation (8, 11, 48). Moreover, when a helper virus is present, it would be expected to form phenotypically mixed viral particles. To test this prediction, we measured viral proteins in cell-free supernatant of nonproducer cells harboring the DuSH provirus, \( \Psi^2 \) cells harboring DuSH DNA, and control \( \Psi^2 \) cells. Cells from these groups were labeled for 3 h with \(^{35}\)S)methionine, and then virions were collected by ultracentrifugation, mixed with unlabeled carrier Moloney vir-

FIG. 4. Myristylation of DuSH Pr60\(^{agg} \) proteins. NIH 3T3 fibroblasts from the \( \Psi^2 \) packaging cell line (lanes 1 and 3) and a clone of \( \Psi^2 \) cells transfected with DuSH neo DNA (lanes 2 and 4) were labeled with \(^1\)H)myristic acid as described in Materials and Methods. The protein extracts were immunoprecipitated with goat anti-gag p30 serum. KD, KIodaldons. On the same gel (data not shown), \(^{35}\)S-labeled control Pr65\(^{agg} \) and Pr60\(^{agg} \) (arrows) comigrated with the two species labeled with \(^1\)H)myristic acid. A 5 to 20% gradient SDS-PAGE gel was used.

FIG. 5. Phosphorylation of DuSH Pr60\(^{agg} \) proteins. NIH 3T3 fibroblasts from the \( \Psi^2 \) packaging cell line (lanes 1 and 3) and a clone of \( \Psi^2 \) cells transfected with DuSH DNA (lanes 2 and 4) were labeled with \(^32\)P, as described in Materials and Methods. Protein extracts were immunoprecipitated by goat anti-gag p30 serum (lanes 1 and 2) or R2 antiserum (lanes 3 and 4). KD, KIodaldons. On the same gel (data not shown), \(^{35}\)S-labeled control Pr65\(^{agg} \) and Pr60\(^{agg} \) comigrated with the two species shown by arrows. A 5 to 20% gradient SDS-PAGE gel was used.
FIG. 6. Subcellular localization of Du5H Pr60\textsuperscript{mew} proteins. NIH 3T3 fibroblasts of the V2 packaging cell line (lane 1) and a clone of V2 cells transfected with Du5H DNA (lanes 2 to 5) were labeled with \textsuperscript{35}S)methionine as described in Materials and Methods. The protein extracts were immunoprecipitated directly (lanes 1 and 2) with goat anti-gag p30 sera and with antisera (R) specific for the p12 region of Duplan virus Pr60\textsuperscript{mew} (see above). In control V2 cells, most of the labeled proteins detected with anti-p30 and anti-p12 were the cleaved products p30 and p12, respectively, and almost no Pr65\textsuperscript{mew} was present (Fig. 7, lanes 4 and 5), as previously reported (8). V2 cells harboring the Du5H provirus released virions containing the Pr60\textsuperscript{mew} molecule, which was easily detected with anti-p30 serum and R antisera (Fig. 7, lanes 1 and 3). As shown above (Fig. 1), anti-p12 serum also reacted poorly with Pr60\textsuperscript{mew} in virions (Fig. 7, lane 2). In virions, Pr60\textsuperscript{mew} was only partially cleaved, generating a small amount of a product, p11, which migrated slightly faster than helper Moloney p12 and was recognized only with R specific antisera (Fig. 7, lane 3).

Interestingly, the levels of helper Moloney Pr65\textsuperscript{mew} were relatively higher in viruses from V2 cells harboring Du5H DNA as detected with both anti-p30 and anti-p12 sera (the ratio of p12 over Pr65\textsuperscript{mew} was >1.1 in virions from V2 cells harboring the Du5H provirus and >10 in virions from V2 cells) (Fig. 7, lanes 2 and 5). Also, the partially cleaved gag product, p40, previously detected in cell extracts (Fig. 2, lanes 12 and 16; 3C, lanes 3 and 5; and 6, lanes 2 and 4) was still as abundant as p30 proteins in virions (Fig. 7, lanes 1) and did not appear to be further cleaved after incorporation into virions. However, in contrast to gag precursors of the other retroviruses, including MuLV, which appear to be sufficient for particle formation (8, 11, 24, 48), no particles were released by cells synthesizing the Du5H Pr60 gag/fusion protein in the absence of helper MuLV (Fig. 7, lane 8). In our experimental conditions, particle release at a level of 5% of that of helper particles would have been easily detected.

The results show that Duplan virus Pr60\textsuperscript{mew} (i) forms phenotypically mixed particles with helper proteins, (ii) is itself only partially cleaved in those phenotypically mixed virions, (iii) interferes with proper cleavage of the helper gag precursor, and (iv) is not sufficient by itself to form particles.

DISCUSSION

The defective Duplan virus was found to be the pathogenic agent of murine acquired immunodeficiency syndrome (1, 4). It was therefore important to identify the protein(s) encoded by its genome and to characterize it. In the present study, we were able to identify a major protein encoded by this virus. It is a gag/fusion protein of about 60 kDa which is slightly smaller than the gag precursors of other helper MuLVs and has a modified p12 region. Like the gag precursors of other MuLVs, Pr60 gag is a polyprotein made of distinct proteins which were recognized with four different sera (anti-p15, anti-p30, anti-p10, and specific anti-p12 R antibodies that we raised). Its reduced size and lack of reaction with anti-gag p12 antibodies were expected because its p12 region is shorter and largely divergent from those of other MuLVs (1). Therefore, this gene product corresponds to the putative protein deduced from our sequence of the viral genome (1).

Chattopadhyay et al. (4) have reported a 60-kDa gag-related protein expressed in cells harboring the defective viral genome. In contrast to our results, they were able to precipitate this protein with anti-p12 antibodies. The discrepancy most likely reflects the anti-p12 antibodies used by the two groups: these may well recognize different epitopes.

The defective Pr60 gag/fusion protein is not cleaved in nonproducer fibroblasts but is partially cleaved in cells superinfected with helper MuLV. This protein was found to be myristylated, phosphorylated, and associated with the cell membrane, like wild-type helper gag precursors. In contrast to these, it is not released on particles into the medium in the absence of helper MuLV proteins, although previous studies have suggested that gag is the only viral gene product needed for budding and particle formation (8, 11, 22, 24, 48) and that processing of the gag precursor does
not even appear to be a prerequisite for the budding process (5, 11, 22, 24, 48). However, when cells producing the defective Pr60 gag/fusion protein were superinfected with a helper MuLV, it was found to be packaged into virions and partially cleaved, as determined by detection of a p11 protein, slightly smaller than p12, with our specific R antibody raised against defective p12 determinants. This indicates that cleavage of Pr60gag can occur, presumably in trans, with the helper protease, although at a reduced rate. This most likely reflects an inappropriate secondary structure of the protein due to its modified p12 region or the inefficiency of protease cleavage in trans, as for the Rous sarcoma virus gag precursor (49), although it does not appear to be the case for other retroviruses. Interestingly, Jones et al. (22) recently reported that a gag–β-galactosidase fusion protein containing MA, p12, and CA determinants was efficiently incorporated into virions and partially cleaved in the presence of helper proteins.

Interestingly, in cells harboring both the defective and helper MuLVs and in virions released from these cells, an additional gag-related protein of approximately 40 kDa was consistently detected. This protein was immunoprecipitated with anti-p10 and anti-p30 sera but reacted poorly with anti-p15 and anti-p12 sera and was not present in cells infected with either virus alone. It most likely represents a partially cleaved gag product. Such a 40-kDa gag intermediate containing p30 and p10 determinants has already been identified in MuLV (33). This p40gag intermediate most likely arises from the helper Pr65gag precursor. Indeed, the relatively low level of Pr60gag found in nonproducer cells remained constant and did not decrease following infection with helper MuLV, as would be expected if Pr60gag were cleaved and processed. Also, the ratio of the amount of p12 over Pr65gag in virions was much higher in cells infected with the helper alone than in cells containing the helper and the defective viruses. If the origin of this 40-kDa protein is indeed the helper Pr65gag precursor, then our results indicate that the defective Pr60gag/fusion protein interferes with cleavage of the helper gag precursor, behaving as a dominant negative mutant, possibly by forming chimeric multimers with the helper gag precursor or somehow inhibiting the protease. Similar multimerization between wild-type and mutated human immunodeficiency virus type 1 gag monomers has been reported to occur before processing, giving interference at the level of or following viral assembly (46).

Although the Pr60gag/fusion protein represents a major gene product of this defective MuLV, requiring more than half of its coding capacity, we have also noticed the presence of another open reading frame at the 3′ end of the genome (1). This putative protein would be made essentially of remnants of pol and env read in alternate reading frames. Since no spliced mRNA was detected in infected fibroblasts in vitro and in infected target cells in vivo (17), this putative protein (25 kDa) would have to be synthesized on the full-length genomic RNA. With the in vitro translation system, we could not detect a 25-kDa protein nor any other protein larger than 60 kDa. Although we cannot exclude the possibility that other pol proteins are encoded by the defective viral genome in infected cells or in reticulocyte extracts at an undetectable level, our results suggest that the 4.2-kb genomic RNA is monocistronic and that stop codons readthrough and ribosomal frameshift do not occur at detectable levels on this genome, as in some other retroviruses (8, 47). Our data suggest that the Pr60gag/fusion protein is likely to be the only gene product of this defective MuLV.

Since this defective virus is pathogenic (1), its major gene product, Pr60gag, which may also be its only encoded protein, is likely to be involved in the pathogenesis of the disease, although our present data do not address this point directly. We previously postulated that this virus behaves as an oncogenic retrovirus on the basis of the fact that it induces oligoclonal expansion of target cells in the absence of virus replication (17). Therefore, the role of the Pr60gag/fusion protein may be that of an oncprotein. The mechanism by which such a protein would participate in cell transformation is totally unclear. Its membrane location suggests that it interacts with other membrane-bound proteins, although it could also interact with other non-membrane-associated cell factors (10). There is no precedent for a direct role of gag proteins in growth dysregulation.

However, indirect evidence for such a role has been reported. The gag regions of several murine retroviruses, such as Moloney, Friend, and Cas-Br-E MuLVs, have been found, by construction of various chimeric MuLVs, to harbor minor determinants of leukemogenicity, sometimes with no apparent effect on the viremia (7, 15, 19, 34–36). Also, the avian osteopetrosis virus has been reported to harbor the major determinant of pathogenicity within its gag region (41). In addition, recently, amino acid residues from the gag amino terminus, fused to the oncprotein v-erbB, have been found to increase the transformation potential of this protein significantly (2). Furthermore, gag proteins are known to interact with cellular factors. The gag p30 protein has been identified as the critical viral protein necessary for Fv-l-mediated cellular restriction of MuLV replication, presumably interacting directly or indirectly with the Fv-l gene product (6, 18, 37). Also, during budding, the Pr65gag precursor is likely to interact with other (unidentified) membrane proteins for specific localization and promotion of budding. However, gag-encoded proteins are still too poorly understood for an explanation of their specific role in each step of the virus cycle. For example, no function has ever been reported for the gag p12 protein, which was, for this reason, excluded from the current nomenclature of retroviral proteins (26). It is intriguing that the most divergent sequence of Duplan defective viral Pr60gag resides within p12, suggesting that it harbors the determinant of pathogenicity for this virus.

Therefore, considering the circumstantial evidence for a role of the gag region in MuLV leukemogenicity and avian osteopetrosis and the dramatic effect of gag residues on v-erbB transformation, we hypothesize that the Pr60gag/fusion protein from the defective Duplan virus is implicated in cell transformation by this virus, possibly behaving as an oncprotein. Our present data on the structural characteristics of this protein should help in testing this hypothesis.

ACKNOWLEDGMENTS

This work was supported by grants to P.J. from the Medical Research Council of Canada and the National Cancer Institute of Canada.

We are grateful to Jolanta Gutkowska for helping with antibody preparation. We thank Marie Bernier for preparing the manuscript.

LITERATURE CITED


