Key words: MuLV envelope proteins/gp70, p15(E), p12(E)/M-MuLV env gene products

Processing of the env Gene Products of Moloney Murine Leukaemia Virus

By VALERIE L. NG, T. GORDON WOOD and RALPH B. ARLINGHAUS

1Department of Tumor Virology, The University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030, U.S.A. and 2National Cancer Institute, Laboratory of DNA Tumor Viruses, Bethesda, Maryland 20205, U.S.A

(Accepted 23 November 1981)

SUMMARY

The initial env gene polyproteins present in Moloney murine leukaemia virus (M-MuLV)-infected NIH/3T3 cells were examined to determine their relationship to each other as well as their role in generating env gene products gp70, p15(E) and p12(E). Steady-state labelling with [3H]glucosamine revealed anti-gp69/71 immunoprecipitable proteins of mol. wt. 93000 (gPr93\text{env}), 83000 (gPr83\text{env}) and 70000 (gp70), whereas similar labelling with [3H]fucose showed only two bands of anti-gp69/71 immunoprecipitable radioactivity migrating in SDS–polyacrylamide gels with gPr93\text{env} and gp70. Pulse-chase experiments employing [3H]leucine labelling instead of labelled sugars failed to detect gPr93\text{env} using similar techniques. The gPr83\text{env} was the only polypeptide detected in 15 min [3H]leucine pulse-labellings, whereas gPr83\text{env}, gp70, p15(E) and p12(E) were detected in chase experiments using appropriate antisera in immunoprecipitation experiments. Pretreatment of infected cells with tunicamycin, an inhibitor of glycosylation, allowed the synthesis of a major band at mol. wt. 62000 (Pr62\text{env}) and a minor band of 73000 mol. wt. at the expense of gPr83\text{env}. In pulse–chase experiments conducted in the presence of tunicamycin, Pr62\text{env} increased during the early chase period but disappeared during the later stages of the chase. No product of Pr62\text{env} was detected. Cation-exchange chromatography of tryptic digests of radioactive tyrosine-labelled gPr83\text{env}, Pr62\text{env} and gp70 showed sequence relationships among the three proteins. Comparison of the two-dimensional fingerprints of [3H]leucine-labelled gPr83\text{env} and the mature proteins gp70 and p12(E) support their precursor–product relationship. Of interest is the observation that gp70 and p12(E) seemed to share a few leucine-containing tryptic peptides. These results provide strong evidence that gPr83\text{env} is the primary product of the env gene which, upon tunicamycin treatment, is synthesized as a subglycosylated protein, Pr62\text{env}. It appears that gPr83\text{env} undergoes further modification of its core oligosaccharide structure as detected by fucosylation to yield gPr93\text{env}. Our inability to detect gPr93\text{env} by [3H]leucine labellings suggests a close chronological relationship between fucosylation and cleavage of the precursor polyprotein, suggesting that cleavage of gPr93\text{env} yields gp70 and p15(E). The latter is further cleaved to yield p12(E) plus a polypeptide containing the C-terminal end of p15(E).

INTRODUCTION

In vitro translation studies (van Zaane et al., 1977; Murphy et al., 1979) combined with heteroduplex mapping (Rothenburg et al., 1978) of murine leukaemia virus RNA have
demonstrated that the envelope proteins of murine leukaemia viruses (MuLV) are translated from a 21S mRNA encoded by the 3' third of the virus genome. The initial env gene product in infected cells is a glycosylated high mol. wt. precursor polyprotein that undergoes further carbohydrate modification and processing to yield the envelope proteins composed of a 70000 mol. wt. glycoprotein termed gp70 and a non-glycosylated polypeptide termed p15(E) (Naso et al., 1976; Karshin et al., 1977; Famulari et al., 1976). Our studies with Rauscher murine leukaemia virus (R-MuLV) have shown that p15(E) is further cleaved to produce p12(E) (Naso et al., 1976; Karshin et al., 1977). p12(E), like p15(E), appears not to be glycosylated (Naso et al., 1976).

Studies focusing on the elucidation of cellular and viral glycosylation systems have demonstrated that glycosylation initially proceeds by 'en bloc' transfer of a large, branched, high mannose-containing oligosaccharide core structure to an exposed asparagine residue within a triplet sequence of asn-X-ser/thr on a protein via a membrane-bound lipid-linked intermediate (Waechter & Lennarz, 1976). Maturation then proceeds by a 'trimming' of the high-mannose oligosaccharide core structure, followed by the addition of sugars such as fucose, galactose and sialic acid, to yield a complex distinct carbohydrate group (Hunt et al., 1978; Tabas et al., 1978; Turco & Robbins, 1979). Glycosylation of proteins via an N-glycosidic linkage can be abolished by the use of tunicamycin (Takatsuki et al., 1971).

Previous studies have probed the structure and arrangement of the murine retrovirus envelope proteins within the glycosylated polyprotein precursor, and have demonstrated the importance of this protein order in virus assembly (Naso et al., 1976; Karshin et al., 1977; Forchhammer & Turnock, 1978; Witte et al., 1977; Leamnson et al., 1977; van de Ven et al., 1977; Witte & Wirth, 1979; Bolognesi et al., 1978; Pinter & Fleissner, 1975; Schultz & Oroszlan, 1979). These reports either lacked detailed structural analyses required to prove a precursor–product relationship between candidate precursors and mature proteins or contained gaps in the pathway leading to the synthesis of mature envelope proteins.

Our studies presented here have focused upon the detailed intracellular processing pathway and the analysis of the primary structure of the Moloney (M)-MuLV envelope gene precursor polyproteins and their place in the overall processing scheme leading to mature envelope proteins. We have identified the primary M-MuLV intracellular env gene product as a glycosylated polyprotein of mol. wt. approx. 83000 (gPr83env), which contains the antigenic and structural determinants of gp70 and p15(E). Also, carbohydrate modification, in the manner of fucose addition of gPr83env, yields an approx. 93000 mol. wt. (gPr93env) polyprotein, which is proposed to be a rapidly processed intermediate that is proteolytically cleaved to yield gp70 and p15(E).

**METHODS**

*Chemicals.* Tunicamycin (lot no. 361-26E-117) was a generous gift of Dr R. E. Hamill, Lilly Research Laboratories, Indianapolis, Ind., U.S.A. Tunicamycin was dissolved in 0·01 M-NaOH at a concentration of 5 mg/ml and stored in 100 μl amounts at -20 °C until further use.

*Cells and virus.* JLS-V16 Swiss mouse embryo fibroblasts chronically infected with R-MuLV were grown as described previously (Syrewicz et al., 1972). NIH/3T3 cells chronically infected with the clone 1 strain of M-MuLV (Fan & Paskind, 1974) were a kind gift of Dr H. Fan and maintained as described previously (Naso et al., 1976). R-MuLV and M-MuLV were purified from culture fluid as described previously (Syrewicz et al., 1972).

*Labelling of cells and virus.* Radioactive labelling of cells and virus was as reported previously (Karshin et al., 1977). For experiments in which radioactively labelled infected cell extracts were obtained from cells treated with tunicamycin, the procedure was as described...
Murine leukaemia virus env gene products

previously (Edwards & Fan, 1979). Cells were allowed to incubate at 37 °C for designated times prior to radioactive labelling in glucose-free minimal essential medium (MEM) supplemented with 10% dialysed foetal calf serum (dFCS), 1 mg/ml sodium pyruvate and 1 µg/ml tunicamycin. Radioactive labelling was then performed in a glucose-free buffered salt solution supplemented with 2% dFCS, 1 µg/ml tunicamycin and the appropriate radioisotope at 100 µCi/ml. Chase incubations were performed in the same medium used for preincubation.

*Immunoprecipitation of virus proteins from cells.* Immunoprecipitation of radioactively labelled virus proteins from infected cell extracts was as reported previously (Ng *et al.*, 1979). Indirect immunoprecipitation was accomplished by using the Cowan I strain of *Staphylococcus aureus* as the second antibody as described previously (Kessler, 1975). Goat antisera to purified R-MuLV structural proteins were obtained from Research Resources, Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md., U.S.A. Antisera were pretreated with an equal volume of mouse cytoplasmic protein as described by Karshin *et al.* (1977).

Anti-rabbit serum prepared against M-MuLV-deduced synthetic (pentadeca) peptide (termed R) (Sutcliffe *et al.*, 1980) was kindly supplied by Dr. R. Lerner, Scripps Institute, La Jolla, Ca., U.S.A. The R sequence represents 15 codons at the end of the env gene (Sutcliffe *et al.*, 1980). Anti-p15(E) (R-MuLV) rabbit serum was obtained from Dr. D. Bolognesi, Duke University, Durham, N.C., U.S.A.

*Polyacrylamide gel electrophoresis (PAGE).* SDS-PAGE was as reported previously (Karshin *et al.*, 1977). Visualization of radioactivity was by fluorography as described previously (Bonner & Laskey, 1974; Laskey & Mills, 1975).

*Purification of virus proteins.* Radioactive virus prepared from 24 h culture fluids was concentrated, disrupted in an 8 M-guanidine·HCl solution as described previously (Karshin *et al.*, 1977) and applied to a Sephacryl S-200 Superfine (Pharmacia) gel matrix in a 1.5 x 90 cm column buffered with 6 M-guanidine·HCl, 0·01 M-dithiothreitol and 0·05 M-HEPES buffer at pH 6. Elution and separation of virus proteins was accomplished by applying a 15 cm head pressure at 22 °C. Fractions were collected (30 min/fraction; 0·7 ml/fraction), monitored for radioactivity, and peak fractions were pooled, dialysed extensively against 0·05 M-NH₄HCO₃ and lyophilized. Further purification was accomplished by subsequent electrophoresis on 11·25% SDS–polyacrylamide gels as described previously (Karshin *et al.*, 1977). Radioactively labelled precursor polyproteins were obtained from cultures pulse-labeled for 15 min with the appropriate isotope at 100 µCi/ml in a balanced salt solution followed by immunoprecipitation and SDS–PAGE as described previously (Karshin *et al.*, 1977).

*Peptide mapping.* Tryptic digestion of purified precursor and mature virus proteins was as described previously (Karshin *et al.*, 1977). Cation-exchange chromatography of radioactive tryptic peptides was performed as described previously (Karshin *et al.*, 1977; Vogt *et al.*, 1975). Two-dimensional tryptic peptide fingerprinting on thin-layer cellulose acetate plates (20 x 20 cm, plastic-backed; Eastman Kodak) was as reported previously (Kopchick *et al.*, 1979).

**Results**

*Intracellular env gene products*

To examine the gene products derived from the env region of the Moloney virus genome, we utilized MuLV anti-gp69/71, anti-p15(E) and anti-R sera. A 15 min pulse-label of M-MuLV-infected cells (Fig. 1, lanes b to e) with [³H]leucine demonstrated a major band of
Fig. 1. Intracellular env gene products in M-MuLV-infected cells. Two subconfluent quart prescription bottles were pulse-labelled for 15 min with 200 μCi/ml [3H]leucine (50 Ci/mmol, New England Nuclear) in 15 ml Hanks' balanced salt solution containing 2% dialysed newborn calf serum. One culture was harvested immediately (lanes a to e) and the other was rinsed once in Hanks' balanced salt solution and then incubated in complete growth medium for 2 h (lanes f to j). Cytoplasmic extracts (5 ml each) were prepared and 1 ml samples were treated with anti-p30 (lanes b and f), anti-gp69/71 (lanes c and g), anti-R (lanes d and h), anti-p15(E) (lanes e and i) and normal goat sera (lanes a and j). The antigen–antibody complexes were collected by means of the Staphylococcal protein A-antibody adsorbent procedure of Kessler (1975). The immunoprecipitates were washed, solubilized, and analysed by SDS–PAGE on a 6 to 12% gel. The fluorogram was exposed for 2 days.

Radioactivity migrating in SDS–polyacrylamide gels at approx. 83000 mol. wt. (termed gPr83env), immunoprecipitable with antisera prepared against gp69/71 (lane c), the synthetic peptide R (lane d) and p15(E) (lane e). These results show that gPr83env shares antigenic determinants with the mature glycoprotein, gp70, p15(E) and the R-containing peptide. A 15 min pulse–2 h chase was performed to determine the fate of gPr83env (lanes f to j). The mature virus glycoprotein gp70 appeared in the chase (lane g) as did p15(E) (lanes h and i) and p12(E) (lane j). This experiment shows that anti-gp69/71 recognized gPr83env and gp70 (lane g); anti-R recognized gPr83env and p15(E) (lane h); anti-p15(E) recognized gPr83env, p15(E) and p12(E) (lane i). We noted that anti-gp69/71 always precipitated a small amount of p15(E) as well.

Steady-state labelling with radioactive sugars (Fig. 2, lanes 3, 4) revealed the appearance of a slower migrating polypeptide with an apparent mol. wt. of approx. 93000 (gPr93env), which labels with both fucose and glucosamine. Pulse-labellings with [3H]leucine failed to detect gPr93env. The mature glycoprotein, gp70, was labelled with both radioactive fucose and glucosamine. Of noticeable exception was the absence of fucose incorporation into gPr83env, although [3H]glucosamine incorporation into gPr83env was observed. This is not surprising as glucosamine residues are found not only in the first two sugar residues linked to the protein asparagine residue, but are also located in the terminal portions of the modified core oligosaccharide structure. Fucose incorporation is indicative of secondary oligosaccharide modification in that fucose addition occurs subsequent to addition and 'trimming' of the oligosaccharide core structure (Waechter & Lennarz, 1976; Hunt et al., 1978; Tabas et al., 1978; Turco & Robbins, 1979; Hubbard & Robbins, 1979).

A similar situation was observed in the R-MuLV-infected cells in that the primary envelope precursor polyprotein (gPr90env) did not incorporate [3H]fucose, whereas the mature
Murine leukaemia virus env gene products

Fig. 2. Analysis of M-MuLV-specific envelope glycoproteins from virus-infected NIH/3T3 cells. Parallel cultures of M-MuLV-infected cells were either pulse-labelled with \(^{3}H\)leucine for 15 min (lane 2), or labelled for 1 h with \(^{3}H\)glucosamine and \(^{3}H\)fucose (lanes 3 and 4 respectively) in Earle's balanced salt solution as described in Methods. Cytoplasmic extracts were prepared and subjected to indirect immunoprecipitation with goat anti-gp69/71 serum as described in Methods. Lane 1 represents a 15 min pulse-label with \(^{3}H\)leucine of R-MuLV-infected cells followed by immunoprecipitation of the cytoplasmic extract with rabbit anti-R-MuLV serum and co-electrophoresed to serve as mol. wt. markers in determining the size of the M-MuLV envelope protein electrophoresis in adjacent lanes. Electrophoresis was performed on a linear 6 to 12% gradient slab gel as described in Methods.

glycoprotein did (Naso et al., 1976; Karshin et al., 1977). An approx. 93000 mol. wt. env-related protein was detected upon radiolabelling R-MuLV-infected cells with \(^{3}H\)fucose and presumably represents a protein analogous to the M-MuLV gPr93\textsuperscript{env}. A higher mol. wt. envelope precursor polyprotein similar to gPr93\textsuperscript{env} that labelled with either \(^{3}H\)leucine or radioactive fucose or glucosamine was observed in Moloney murine sarcoma virus (M-MuSV)-infected cells (Wood et al., 1980).

Effect of tunicamycin treatment on the env precursor polyprotein

Recent studies have demonstrated that tunicamycin, a naturally occurring antibiotic, can inhibit glycosylation (Mahoney & Duskin, 1979; Hart et al., 1979). Tunicamycin has been used to inhibit glycosylation in MuLV-infected cells. A subglycosylated form of the initial env gene product that migrates faster in SDS-polyacrylamide gels with an apparent mol. wt. loss of 20000 as compared with its glycosylated counterpart has been observed (Witte & Wirth, 1979; Schultz & Oroszlan, 1979).

We examined the effect of the length of preincubation in the presence of tunicamycin (1 \(\mu g/ml\)) on the synthesis of envelope precursor polyproteins (Fig. 3). Increased preincubation times of both M-MuLV- and R-MuLV-infected cells produced a shift of radioactivity from the glycosylated env precursor polyprotein to lower mol. wt. protein which presumably represents the unglycosylated or subglycosylated env precursor polyprotein. In M-MuLV-infected cells (Fig. 3a), a shift of anti-gp69/71 immunoprecipitable radioactivity from gPr83\textsuperscript{env} to Pr62\textsuperscript{env} was observed with increased times of preincubation in the presence of
V. L. Ng, T. G. Wood and R. B. Arlinghaus

Fig. 3. Effect of the length of time of preincubation in the presence of tunicamycin on the synthesis of M-MuLV and R-MuLV env precursor polyproteins. Parallel cultures of (a) M-MuLV- and (b) R-MuLV-infected cells were subjected to preincubation periods of 15, 30, 60, 120 and 240 min in the presence of 1 μg/ml tunicamycin (lanes 2 to 6 respectively) after which a 15 min pulse-label with [3H]leucine was performed as described in Methods. Cell extracts were prepared and indirectly immunoprecipitated with goat anti-gp69/71 serum as described in Methods. Equal cell volumes of immunoprecipitates were analysed on 6 to 12% linear gradient SDS-polyacrylamide gels and processed for fluorography as described in Methods. Radiolabelled M-MuLV and R-MuLV proteins were co-electrophoresed in lane 1 in (a and b) to provide mol. wt. markers.

tunicamycin. A less prominent band migrating at approx. 73 000 mol. wt. [(g)Pr73env] also appeared with increasing exposure to tunicamycin.

R-MuLV-infected cells also contained a lower mol. wt. env precursor polyprotein upon increased length of preincubation time in the presence of tunicamycin (Fig. 3b). Most marked was the appearance of a triplet at approx. 68 000 to 70 000 mol. wt. that presumably corresponds to the unglycosylated R-MuLV env precursor. Schultz & Oroszlan (1979) reported that the apparently unglycosylated R-MuLV env gene was a single entity of 68 000 daltons, and Murphy et al. (1979) have shown that the initial in vitro translation product of the intracellular env gene mRNA is a polypeptide of about 68 000 daltons.

Some synthesis of the glycosylated env precursor polyprotein of R-MuLV and M-MuLV was still observed following 4 h of preincubation in the presence of 1μg/ml tunicamycin. In an attempt to achieve complete blockage, preincubation of M-MuLV- and R-MuLV-infected cells for 4 h in the presence of 100 μg/ml tunicamycin was performed. The results showed a small amount of gPr83env and gPr90env synthesis respectively, arguing that total blockage of glycosylation of env precursor polyproteins was not achieved by the drug (not shown).

Pulse–chase kinetics of the M-MuLV env precursor made in the presence or absence of tunicamycin

We were interested in determining the fate of the subglycosylated form of the env precursor relative to gPr83env during a pulse–chase kinetics study in which tunicamycin was present. Fig. 4(a) represents a control pulse–chase experiment in which the chase kinetics of [3H]leucine-labelled anti-gp69/71 immunoprecipitable material was observed. Radioactivity corresponding to gPr83env from a 15 min pulse-label disappeared very slowly in a chase experiment, and a significant amount of radioactivity was still readily detected in gPr83env following 4 h of chase incubation. Radioactivity in gp70 appeared following 30 min of chase incubation, but did not accumulate within the infected cell to any appreciable extent. Similar findings were observed in the R-MuLV system (Naso et al., 1976). We noted that no [3H]leucine radioactivity in a band corresponding to gPr93env was observed, although
**Murine leukaemia virus env gene products**

Fig. 4. Pulse-chase kinetics of the M-MuLV env precursor polyprotein in the presence or absence of tunicamycin. (a) Parallel cultures of M-MuLV-infected cells were subjected to a 15 min pulse-labelling with [3H]leucine in the absence of tunicamycin, after which chase incubations of 0 (lane 2), 30 (lane 3), 60 (lane 4), 120 (lane 5) and 240 min (lane 6) were performed. Lanes 1 and 7 show R-MuLV-infected cell extracts as markers: lane 1, 15 min pulse; lane 7, 15 min pulse–2 h chase. Cytoplasmic extracts were prepared, immunoprecipitated with anti-gp69/71 serum, and equal cell volumes were analysed on a 6 to 12% linear gradient SDS–polyacrylamide gel. (b) Parallel cultures of M-MuLV-infected cells were first subjected to a 4 h preincubation in glucose-free medium containing 1 μg/ml tunicamycin, after which a 15 min pulse-label with [3H]leucine was performed and subsequent chase incubations of 0 (lane 2), 15 (lane 3) 30 (lane 4), 60 (lane 5), 120 (lane 6) and 240 min (lane 7) were allowed with tunicamycin present at a concentration of 1 μg/ml during all times. Lanes 1 and 8 show M-MuLV-infected cell extracts pulse-labelled for 15 min. Cytoplasmic extracts were processed and analysed as in (a).

gPr93env was detected with [3H]glucosamine and [3H]fucose labelling (see Fig. 2). We interpret these results as indicative of a tight coupling of the carbohydrate modification event(s) and proteolytic cleavage of the precursor polyprotein to yield gp70 and p15(E).
Fig. 5. Cation-exchange chromatography of tyrosine-containing tryptic peptides of M-MuLV env gene products. [14C]tyrosine-labelled M-MuLV gp70 was purified from fluid virus subjected to gel filtration in 6 M-guanidine-HCl and further purified by SDS-PAGE as described in Methods. [3H]tyrosine-labelled gPr83env was obtained by pulse-labeling M-MuLV-infected cells for 15 min followed by indirect immunoprecipitation with anti-gp69/71 serum. [3H]tyrosine-labelled Pr62env was obtained in a similar manner except that M-MuLV-infected cells were first subjected to a 4 h preincubation in glucose-free medium containing 1 μg/ml tunicamycin before pulse-labeling in the presence of tunicamycin. The proteins were purified by SDS-PAGE, subjected to tryptic digestion, and the tryptic peptides were separated by cation-exchange chromatography. The arrow on the abscissa marks the end of the pyridine-acetate elution gradient buffer and the start of the 2 M-pyridine-acetate wash. (a) Co-chromatography of [3H]tyrosine-labelled gPr83env (—) and [14C]tyrosine-labelled gp70 (---); (b) co-chromatography of [3H]tyrosine-labelled Pr62env (—) and [14C]tyrosine-labelled gp70 (---).

Fig. 4 (b) represents an experiment in which M-MuLV-infected cells were subjected to a 4 h preincubation and subsequent pulse-chase analysis with [3H]leucine in the presence of tunicamycin (1 μg/ml). During a 15 min pulse-label, a major band of radioactivity corresponding to Pr62env was observed. Minor amounts of radioactivity in bands migrating both slower [(g)Pr73env] and faster than Pr62env were also observed. Pr62env seemed to disappear very slowly since radioactivity in this band was still detected after 4 h of chase incubation. No new anti-gp69/71 immunoprecipitable proteins were detected during the chase incubations, suggesting that the subglycosylated Pr62env does not give rise to a subglycosylated form of gp70. In a parallel experiment, in which the chase was done in the absence of tunicamycin, similar results were obtained. Of particular interest in the latter experiment was the absence of gPr83env, suggesting that gPr83env may only be glycosylated during its synthesis.
Fig. 6. Two-dimensional fingerprint analysis of the leucine-containing tryptic peptides of the M-MuLV env gene products. \(^{3}H\)leucine-labelled gPr83\(^{env}\) was obtained as described in Fig. 5. \(^{3}H\)leucine-labelled gp70 and \(^{14}C\)leucine-labelled p15(E) were purified from radioactively labelled fluid virus subjected to 6 M-guanidine–HCl gel filtration and subsequent SDS–PAGE as described in Methods. Samples were processed as in Fig. 5, and subjected to two-dimensional fingerprint analysis. Electrophoresis was from left to right and chromatography was from bottom to top. \(^{3}H\)leucine tryptic peptides of M-MuLV: (a) gPr83\(^{env}\) (83 000 ct/min applied, exposed 4 weeks); (b) gp70 virus (70 000 ct/min applied, exposed 4 weeks); (c) \(^{14}C\)leucine-labelled tryptic peptides of p12(E) (10 000 ct/min applied, exposed 4 weeks).
Analysis of the primary structure of the M-MuLV env gene product by cation-exchange chromatography of tyrosine tryptic peptides

To further investigate the protein backbone of the M-MuLV env gene product, we obtained \[^{14}\text{C}]\text{tyrosine-labelled } \text{gp70}\) from purified fluid virus, \[^{3}\text{H}]\text{tyrosine-labelled } \text{gPr83}^{\text{env}}\) from pulse-labelled M-MuLV-infected cells and \[^{3}\text{H}]\text{tyrosine-labelled } \text{Pr62}^{\text{env}}\) from pulse-labelled cells pretreated with tunicamycin as described in Methods. The proteins were subjected to tryptic digestion and the tryptic peptides separated by cation-exchange chromatography as described in Methods.

The tryptic peptide patterns of the glycosylated and subglycosylated env gene product were compared with that of mature M-MuLV gp70 (Fig. 5). In comparing the tryptic peptide profiles of M-MuLV gPr83\(^{env}\) and Pr62\(^{env}\), the similarity in peptide profiles was apparent. It should be pointed out that the patterns of peptides eluting in the 2 M-pyridine wash indicated by the arrows in Fig. 5 were variable. Some major differences were, however, observed. For example, the major tyrosine-labelled peptide eluting at fraction 126 was observed in Pr62\(^{env}\) but not in gPr83\(^{env}\). Another important difference was the near absence of radioactivity appearing in the void volume (fractions 1 to 20) in the Pr62\(^{env}\) profile as compared with the amount of radioactivity voiding in the profile of gPr83\(^{env}\). Previous studies involving elution of tryptic peptides labelled with radioactive sugars have demonstrated that nearly all the radioactivity appears in the void volume (L. Arcement & R. B. Arlinghaus, unpublished results).

Analysis of two-dimensional fingerprints of leucine-containing tryptic peptides of the M-MuLV Pr83\(^{env}\), gp70 and p12(E)

Previously published results concerning M-MuLV gPr83\(^{env}\) have established that it shares antigenic determinants and peptide sequences with gp70 (Witte & Wirth, 1979), but no information is available on the presence of p15(E)/p12(E) in M-MuLV gPr83\(^{env}\). Naso et al. (1976) previously reported the presence of methionine-containing tryptic peptides of p15(E)/p12(E) within gPr90\(^{env}\) for the R-MuLV system. However, no detectable R-MuLV gp70-specific methionine-containing tryptic peptides were found within the precursor. Karshin et al. (1977) were able to demonstrate the presence of R-MuLV gp70 and p15(E) tyrosine tryptic peptides within R-MuLV gPr90\(^{env}\). Because p15(E) is present in only minor amounts in murine retroviruses (Naso et al., 1976; Karshin et al., 1977), we chose to digest p12(E), a major virus protein, with trypsin. We also had difficulty in obtaining tyrosine-labelled p12(E) from M-MuLV, but were able to label p12(E) with \[^{3}\text{H}]\text{leucine}.

Two-dimensional fingerprints of \[^{3}\text{H}]\text{leucine-labelled } \text{gPr83}^{\text{env}}, \text{gp70} \text{and } \text{p12(E)}\) are shown in Fig. 6. A total of 21 leucine-containing tryptic peptides were resolved in gPr83\(^{env}\). It is of interest that a subset of these spots was derived either from p12(E) (spots 5, 8b, 10) or gp70 (spots 1 to 4, 6 to 8a, 9 to 17 and 21). We noted the appearance of peptide spots 10, 11 and 13 in both the gp70 and p12(E) fingerprints. In addition, minor amounts of gp70 spots 12, 15, 16 and 17 were also detected in p12(E).

Of interest is the observation that spot 1 in the gp70 fingerprint contains a large proportion of the total radioactivity and this might mask the presence of spot 2 in the gp70 fingerprint. Spot 1 migrates in this separation system in the manner observed with tryptic peptides labelled with \[^{3}\text{H}]\text{glucosamine} or \[^{3}\text{H}]\text{fucose} (T. G. Wood & R. Arlinghaus, unpublished results), and is implicated as a glycosylated peptide(s). The large amount of radioactivity may be attributable to the trypsin-resistant nature of glycosylated peptides conferred by bulky oligosaccharide side-chains (Witte & Wirth, 1979). In addition, a gp70-unique spot, spot 20, was observed that was not present in the gPr83\(^{env}\) fingerprint.
**DISCUSSION**

We have identified and partially characterized three size classes of intracellular polyproteins that share sequences with M-MuLV gp70 and p15(E)/p12(E). They consist of two glycosylated polyproteins of approx. 83,000 mol. wt. (gPr83\textsuperscript{env}) and approx. 93,000 mol. wt. (gPr93\textsuperscript{env}), and a subglycosylated polyprotein of approx. 62,000 mol. wt. (Pr62\textsuperscript{env}). The gPr83\textsuperscript{env} appeared to be the major intracellular polyprotein precursor of the mature env gene products, as detected by pulse-labeling M-MuLV-infected cells followed by immunoprecipitation with anti-gp69/71, anti-p15(E) and anti-R sera. Incorporation of radioactive glucosamine demonstrated the glycosylated nature of gPr83\textsuperscript{env}, but lack of radioactive fucose incorporation demonstrated the fucose-deficient nature of gPr83\textsuperscript{env}. The gPr83\textsuperscript{env} appeared to be a relatively unstable intracellular protein since long chase experiments clearly showed a decrease in gPr83\textsuperscript{env} and an increase in gp70. However, considerable amounts of radioactivity in gPr83\textsuperscript{env} were detectable following 4 h of chase incubation.

The use of antisera to p15(E) and the R peptide has allowed us to provide more definitive information on the nature of p15(E) processing. The R peptide is a pentadeca peptide sequence representing the last 15 codons at the 3' end of the env gene (Sutcliffe et al., 1980). Our studies indicate that the R peptide shares antigenic determinants with gPr83\textsuperscript{env} and p15(E) but not p12(E), confirming earlier studies by Lerner and colleagues (Sutcliffe et al., 1980; Green et al., 1981). Thus it appears that an R-containing polypeptide and p12(E) are produced as a result of cleavage of p15(E).

Our peptide mapping studies have demonstrated the presence of gp70 and p12(E) leucine-containing tryptic peptides within gPr83\textsuperscript{env}, supporting their precursor-product relationship. However, the observation of leucine-containing tryptic peptide spots 10, 11, 13, and possibly 12, 15, 16 and 17 in both gp70 and p12(E) fingerprints was surprising. These peptide spots appeared to co-migrate upon superimposition of the fingerprints, and may merely reflect a lack of resolution of peptides with different structure within our separation system. Alternatively, contamination of our purified protein preparations may have occurred, such that fragments of gp70 would co-purify with p12(E) through guanidine–HCl gel filtration and subsequent SDS–PAGE. It is possible, however, that these peptides detected within both gp70 and p12(E) may indeed reflect shared structural determinants.

The majority of leucine tryptic peptides in gPr83\textsuperscript{env} could be accounted for in the maps of gp70 and p12(E). We were able to identify two p12(E)-specific peptides (spots 5, 8b) within gPr83\textsuperscript{env}. The remainder of the gPr83\textsuperscript{env} leucine tryptic peptides, with the exception of spots 18 and 19, were also present in gp70. Spots 18 and 19 could conceivably represent p15(E)-specific sequences found in the R-containing peptide but not found in its cleavage product, p12(E). It is also possible that one or both of these tryptic peptides might be present in gp70, but are not detected because further glycosylation has rendered them trypsin-resistant. Indeed, a large amount of applied radioactivity in the gp70 fingerprint appeared to be trypsin-resistant as represented by the increased material in spot 1. The gp70 exhibited a unique leucine tryptic peptide (spot 20) not present in gPr83\textsuperscript{env}. Spot 20 could represent a junction peptide between gp70 and p15(E) within gPr83\textsuperscript{env}, such that the trypsin cleavage site in gPr83\textsuperscript{env} would not correspond to the site of cleavage between gp70 and p15(E).

A higher mol. wt. glycosylated polyprotein of approx. 93,000 (gPr93\textsuperscript{env}) immuno-precipitable with anti-gp69/71 serum was a novel observation in MuLV-infected cells. We have been able to identify gPr93\textsuperscript{env} only if radioactive sugars (i.e. glucosamine and fucose) were used as labelling substrates; a labelling with radioactive amino acids using our usual labelling conditions failed to reveal gPr93\textsuperscript{env} within M-MuLV-infected cells. Addition of a higher concentration of radioactive amino acid to the culture should facilitate the detection of gPr93\textsuperscript{env}, but this possibility has not been tested. Mature gp70, like gPr93\textsuperscript{env}, was also shown to contain glucosamine and fucose residues. Therefore, we conclude that modification of the
core oligosaccharide side-chains of gPr83\textsuperscript{env} in the manner of fucose addition must be an event closely linked to cleavage of the precursor to yield gp70 and p15(E). Indeed, our data support a hypothesis put forth by Witte & Wirth (1979) in which they suggested that carbohydrate modification of the precursor polyprotein was closely timed to proteolytic processing. This higher mol. wt. fucosylated precursor polyprotein (gPr93\textsuperscript{env}) has also been detected in R-MuLV-infected cells. In addition, a higher mol. wt. glycosylated env-related polyprotein identical in size to gPr93\textsuperscript{env} has been detected in M-MuSV/MuLV-infected cells after labelling with either \(^{3}\text{H}\)leucine or radioactive sugars. Because M-MuSV-124 has a large deletion in the env region largely occupied by the src gene (Hu et al., 1977), the env gene product must arise from the co-infecting helper MuLV (Wood et al., 1980). In this same study, it was found that gPr93\textsuperscript{env} transiently appeared in chase experiments consistent with its role as an intermediate arising from the primary precursor gPr83\textsuperscript{env}. It would appear that carbohydrate modification of the initial glycosylated env gene precursor polyprotein in the manner of fucose addition to yield a higher mol. wt. env-related polyprotein precursor (gPr93\textsuperscript{env}) is a common step in the overall MuLV biosynthetic pathway generating gp70 and p15(E)/p12(E).

The use of tunicamycin (1 \(\mu\)g/ml), an inhibitor of glycosylation (Mahoney & Duskin, 1979; Hart et al., 1979), revealed anti-gp69/71 immunoprecipitable proteins of approx. 62,000 mol. wt. (Pr62\textsuperscript{env}) and approx. 68,000 to 70,000 mol. wt. (Pr63/69/70\textsuperscript{env}) in M-MuLV- and R-MuLV-infected cells respectively. We noted that a 100% inhibition of glycosylation was not obtained even with preincubation of cells for 4 h in the presence of 100 \(\mu\)g/ml tunicamycin. The reason(s) for this lack of complete blockage is unknown. We thus refer to M-MuLV Pr62\textsuperscript{env} and R-MuLV Pr68/69/70\textsuperscript{env} as subglycosylated forms of gPr83\textsuperscript{env} and gPr90\textsuperscript{env} respectively, in that we have not shown the complete absence of sugar residues on the proteins synthesized in the presence of tunicamycin.

Pulse-chase studies of Pr62\textsuperscript{env} revealed similar chase kinetics relative to gPr83\textsuperscript{env} in that both polyproteins gradually disappeared as the chase interval was increased. However, no new anti-gp69/71 immunoprecipitable proteins were detectable during chase incubation of
Pr62\textsuperscript{env}. The disappearance of Pr62\textsuperscript{env} from the infected cell probably results from non-specific degradation within the infected cell.

Peptide mapping of gPr83\textsuperscript{env} as compared with Pr62\textsuperscript{env} revealed a high degree of similarity. Tryptic peptides labelled with radioactive sugars elute in the initial flow-through fractions during ion-exchange chromatography (V. L. Ng et al., unpublished results), suggesting a highly acidic nature of these peptides as conferred by the carbohydrate moieties. The relatively small amount of radioactivity in the initial fractions of the Pr62\textsuperscript{env} profile compared with either gp70 or gPr83\textsuperscript{env} is indirect evidence of the subglycosylated nature of this molecule. Of interest is that Pr62\textsuperscript{env} contained a major tryptic peptide (fraction 126) not within the gPr83\textsuperscript{env} profile. Since Pr62\textsuperscript{env} represents a subglycosylated form of gPr83\textsuperscript{env}, the peptide eluting at fraction 126 thus becomes a likely candidate for containing the site(s) of glycosylation essential in the generation of gPr83\textsuperscript{env}. The glycosylated peptide analogous to fraction 126 in Pr62\textsuperscript{env} might possibly elute in the flow-through. A similar detection of a new tyrosine tryptic peptide in the R-MuLV unglycosylated \textit{env} polyprotein precursor, Pr68\textsuperscript{env}, synthesized by \textit{in vitro} translation of 22S cellular RNA upon comparison with \textit{in vivo} synthesized gPr90\textsuperscript{env}, has been made (Murphy et al., 1979).

Analysis of glycoprotein synthesis by others has demonstrated that glycosylation can occur on the nascent polypeptide chain (Rothman & Lodish, 1977). Indeed, in these studies, if glycosylation enzymes were withheld during synthesis of the apoprotein backbone, glycosylation of the completed apoprotein would not occur upon addition of the appropriate glycosylating enzymes (Rothman & Lodish, 1977), demonstrating a synchrony between protein synthesis and glycosylation. Thus, our identification of gPr83\textsuperscript{env} as the major primary env gene product in M-MuLV-infected cells demonstrates translation of the intracellular env mRNA concurrent with glycosylation. If we treated cells with tunicamycin, an inhibitor of glycosylation, we mainly detected a lower mol. wt. env gene product (Pr62\textsuperscript{env}) representing a subglycosylated form of gPr83\textsuperscript{env}. Chase incubation of Pr62\textsuperscript{env} in the absence of the drug never yielded gPr83\textsuperscript{env}, further demonstrating that if the env gene product was not glycosylated during protein synthesis it would not be glycosylated post-translationally. Indeed, our failure to detect Pr62\textsuperscript{env} during a pulse-label of infected cells in the absence of tunicamycin is indicative of the close coupling of glycosylation and translation, and that only a perturbation of such events (e.g. tunicamycin treatment) will allow detection of subglycosylated Pr62\textsuperscript{env}.

The above evidence is consistent with the model shown in Fig. 7. In this model, the initial gene product of the env gene mRNA is gPr83\textsuperscript{env}. A block of glycosylation yields a dead-end product, Pr62\textsuperscript{env}. If glycosylation is not interfered with, gPr83\textsuperscript{env} can be further glycosylated as measured by fucosylation to yield a transient intermediate, gPr93\textsuperscript{env}, that is rapidly cleaved to yield gp70 and p15(E). Viral p15(E) is then further processed to yield p12(E) and the R-containing peptide. The gPr93\textsuperscript{env} is visualized as an obligate precursor, but it is conceivable that it represents an alternate pathway since our results do not rule out the possibility of direct cleavage of gPr83\textsuperscript{env} followed by fucosylation of gp70.

The MuLV envelope glycoproteins play an important role in virus assembly and infectivity. Indeed, the virus envelope proteins determine the host range of the virus, and thus play an important role in virus infection and the expression of oncogenic virus genes. Recombinant events between non-ecotropic and ecotropic viruses can be detected in virus RNAs specifying env gene material (Faller et al., 1978). In addition, deletion of a portion of the M-MuLV genome and acquisition of new genetic material to yield a murine sarcoma virus (MuSV), as in the case of Moloney MuSV-124, involves loss of some if not all of the M-MuLV env gene, as demonstrated by heteroduplex mapping (Hu et al., 1977; Donoghue et al., 1979). The primary structural analyses we have shown here of the M-MuLV env gene proteins will aid us in determining whether a residual portion of the env gene is translated and becomes connected to the src gene product encoded by M-MuSV-124.
We thank R. B. Naso for valuable discussions. We thank Karen Draeger and James J. Syrewicz for excellent technical assistance, and Rebecca Bazer for manuscript preparation. V.L.N. was supported by a Rosalie B. Hite predoctoral fellowship in cancer research. This work was supported by grants (G-429) from The Robert A. Welch Foundation and NIH (CA-25465 and CA-16672).

REFERENCES


Murine leukaemia virus env gene products


(Received 5 August 1981)