Expression and Purification of Recombinant Marek's Disease Virus Serotype 1 Specific Phosphorylated Protein pp38 in *E. coli*

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ABSTRACT. Phosphorylated protein pp38 is the only protein that is detected in the Marek's disease (MD) lymphoma caused by MD virus serotype 1 (MDV-1) and lymphoblastoid cell lines. In this study, a recombinant protein coded for by the almost entire open reading frame of the MDV-1 pp38 cDNA was produced in *E. coli* and purified by affinity chromatography. Prior to the expression and purification of the protein, cDNA containing the entire coding region for pp38 was cloned and its nucleotide sequence was determined. Immunoblot analysis indicated that the expressed recombinant protein electrophoresed close to that of the pp38 in infected cells. The difference in mobility of the purified recombinant and the pp38 in infected cells corresponded to a fusion peptide. The recombinant pp38 may be of interest for function analyses and the diagnostic use of pp38.—**KEY WORDS:** Marek's disease virus, M21 monoclonal antibody, Ni-tag, phosphorylated protein, recombinant pp38.

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Marek's disease virus serotype 1 (MDV-1) is known to cause T-cell lymphoma in chickens. T-cell lymphomas occur after the establishment of latency in MDV-infected chickens (MD lymphoma) [2]. The virus protein expressed in the tumor or transformed cells is a candidate for the gene-controlling protein which maintains the state of transformation. Phosphorylated protein pp38 which appeared to be identical to the pp39/36 protein reported by Nakajima *et al.* [14] is the only protein that was detected in MD lymphoma and lymphoblastoid cell lines [10].

Bradley *et al.* [1] reported a candidate for the transcription unit of pp38 in BamHI-H fragment. Cui *et al.* [6] determined the genomic sequence of the entire coding region for pp38. Chen *et al.* [3] further analysed the structure of the partial cDNA. Although the entire coding region was sequenced, no cDNA containing the entire coding region was isolated. The entire open reading frame (ORF) codes for this protein give a calculated molecular weight of 31,169. In contrast, the main band on Western blotting was reported to be 38 or 39 kDa [5, 14]. Recombinant pp38 protein expressed in *E. coli* could provide information about this anomalous mobility, because a protein expressed in *E. coli* is rarely modified. Recombinant pp38, which has almost the same primary structure as pp38 in virus-infected cells, would be used in experiments for functional inspection such as a DNA-binding assay or protein interaction assay [4, 12, 13, 16]. The recombinant polypeptide also can be used for

titration of anti-MDV antibodies in chicken serum [17, 21].

In this study, we cloned a cDNA containing the entire ORF from MDV-1-infected cells and determined its nucleotide sequence. With this cDNA, the entire ORF was expressed as a recombinant protein fused with an affinity tag sequence in *E. coli* and purified by Ni-affinity chromatography. The aim of this study is preparation of a recombinant pp38 which may be used for structure-function analyses of the pp38 in infected cells.

MATERIALS AND METHODS

The non-virulent MDV strain CVI-988, which belongs to MDV-1, was obtained from Kyoritsu-shozi, Tokyo. The viruses were grown in chicken embryo fibroblasts (CEF). Procedures for the growth and maintenance of cells were described previously [15]. The DNA clone of the genomic fragment of BamHI-H was a gift from Dr. M. Nonoyama [8].

A cDNA library was constructed in the λ gt11 phage from polyadenylated RNA isolated from polyadenylated RNA isolated from CVI-988-infected CEF, and was screened by plaque hybridization with the EcoRI-X genome fragment prepared from the BamHI-H genome fragment of MDV [8]. Hybridizing plaques were purified by three subsequent screening steps. Two clones of phage DNA containing the largest cDNA insert were subcloned into Bluescript KS+ and SK+ plasmids (Stratagene, LaJolla, U.S.A.) and both the clones were sequenced with a standard deletion protocol using exonuclease III (Toyo Boseki, Osaka) and mung bean nuclease (Toyo Boseki, Osaka) [18]. Sequencing was performed by the dideoxy-

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10      20      30      40      50      60      70      80      90
CCTCTCGTCGGACGGGAGCGCGGTATAGGATAAGAGATCACAAAAAGCGAGACCTGGATCGAACGCAACGTCCTCGTCCCGGTGT
100     110     120     130     140     150     160     170     180
AATCGTPTGGAACTCTCTCGCCTGATCGGTGGTGTAAACCGTGAGGATATTTTAGTPTTATGTACCAATTTCCGGTTGCTTTATATATATTT
190     200     210     220     230     240     250     260     270
CCCACCCATCGTTTTTCTTTATAACATAGTTTGACCCCTCTCGGCATCACAGATGGCACCCCTCCCTCCACTCATGACCCACAACCGGATT
280     290     300     310     320     330     340     350     360
TGTTTTTCATCTTCAACCCACAGCCATCCTTGCTTTTCIGCCCGCACCGCACGCTTTGCTCGTCCCGCGTGAAGATCGGCAGGGGGTG
370     380     390     400     410     420     430     440     450
ATGGAATTCGAAAGCAGAACACGAAGGGCTGACGGCGTCTTGGGTGCGCCCCGCTCCCGAGGTGGAAAGGGGCGGAGGCCCGCAGGG
MetGluPheGluAlaGluHisGluGlyLeuThrAlaSerTrpValAlaProAlaProGlnGlyGlyLysGlyAlaGluGlyArgAlaGly
460     470     480     490     500     510     520     530     540
GTCGCCGACGAGGCGAGGCGATGGCAAAACAGAAAGCGGAATGCGCCGAGGACGGCGAGAAATGCGGGGACGCCGAGATGAGCGCTTTGGAT
ValAlaAspGluAlaGlyHisGlyLysThrGluAlaGluCysAlaGluAspGlyGluLysCysGlyAspAlaGluMetSerAlaLeuAsp
550     560     570     580     590     600     610     620     630
CGGGTCCAGAGGGACCGGTGGAGATTGAGTTCTCCGCCCTCACTCTGGACTCACGGGGAAGGGGGCTATTCCAATAAACGGTGATGGG
ArgValGlnArgAspArgTrpArgPheSerSerProProProHisSerGlyValThrGlyLysGlyAlaIleProIleLysGlyAspGly
640     650     660     670     680     690     700     710     720
Gln
A
AAGGCGATAGAAATGCCAGGAGCTAACCGGAGAGGGAGAGTGGCTGTACCGTGGGGGAGCTACCGCTGAGCCCGGAGGTGAGGGAAT
LysAlaIleGluCysGlnGluLeuThrGlyGluGlyGluTrpLeuSerArgTrpGlyGluLeuProProGluProArgArgSerGlyAsn
730     740     750     760     770     780     790     800     810
GAACATCTTGACGAAAGTCGGTATGCGAAACAAACCGAAAGGGGTAGCTCTACGGGGAAGAAGAGGGAGATGGTATGAAGCAGATGGGG
GluHisLeuAspGluSerArgTyrAlaLysGlnThrGluArgGlySerSerThrGlyLysGluGluGlyAspGlyMetLysGlnMetGly
820     830     840     850     860     870     880     890     900
GAGCTTGCCAGCAGTCCCAAGGAGGAACATATGCGGACTTGCTTGTCGAAGCAGAGCAAGCTGTGTACATTCGGTTCGCGCATTATG
GluLeuAlaGlnGlnCysGluGlyGlyThrTyrAlaAspLeuLeuValGluAlaGluGlnAlaValValHisSerValArgAlaLeuMet
910     920     930     940     950     960     970     980     990
CTGGCCGAAAGACAAACCAATATATTTGGGGGAGCATTTGAATAAAAAACGGGTCTTGTACAAACCCCGTACTATTCTATCCGTC
LeuAlaGluArgGlnAsnProAsnIleLeuGlyGluHisLeuAsnLysLysArgValLeuValGlnArgProArgThrIleLeuSerVal
1000    1010    1020    1030    1040    1050    1060    1070    1080
GAGTCAGAGAATGCAACAATGCGTTCTTATATGCTGGTTACATTGATCTGTCTCTGCAAAATCATTATTACTAGGATCGTCATGTCTATT
GluSerGluAsnAlaThrMetArgSerTyrMetLeuValThrLeuIleCysSerAlaLysSerLeuLeuLeuGlySerCysMetSerPhe
1090    1100    1110    1120    1130    1140    1150    1160    1170
TTTCGCTGGTATGTTAGTTCGGTAGAACGCGCAGACGTAAAAACACCATTTATGGGATACGTGTATGTTTGTTAATGGCTTTCTGTGCAGGCATT
PheAlaGlyMetLeuValGlyArgThrAlaAspValLysThrProLeuTrpAspThrValCysLeuLeuMetAlaPheCysAlaGlyIle
1180    1190    1200    1210    1220    1230    1240    1250    1260
TTCGTTGGGGAGTGGATTCTGGGAGGTGGAATCTGGTGAACAAAAATCTGAATCAAATTTAAATTTAATACAGTGTAGCCGTACCCGAC
ValValGlyGlyValAlaAspSerGlyGluValGluSerGlyGluThrLysSerGluSerAsn
1270    1280    1290    1300    1310    1320    1330    1340    1350
GTTGGAGCGGAGATTAAGCGAATTCTACCTTTACGAATATTTGGTGCAGACAAAGACCAAAAAATGGAAAAATGGACAGCTGCAGCAGCA
1360    1370    1380    1390    1400    1410    1420    1430    1440
AAGTCTCGAATTTGGATGCAGATGCCGTCTTATATACCGAGACTATCTCCCAACCAATCGAGGAAGAACCCTGTCTTTCAGATATTGATGA
1450    1460    1470    1480    1490    1500    1510    1520    1530
ACAATCAGAATATATTCAATTTACAATTCGAATCGGTTACAGATACAATAATTTCCCACTGTTGCCCATATACGATGATCAGTTGACCC
1540    1550    1560    1570    1580    1590    1600    1610    1620
ACCCCTTCATACGATTCCTTATCCCGGATACATAATGTTTAAACAATCTGAAAGTTGCGCAGAAGTTGACTTTCGCTTTTATCATTCGACA
1630    1640    1650    1660    1670    1680    1690    1700    1710
TGATGGATGTGCGATCGCTACATTTAATACTTTTCTGACGGTAGTTCTGCAACCCCTTGTAACATTATATCACAGAAACATAATTGAC
1720    1730    1740    1750
GTATCTGATACAATAAATATGCACCTACCAAAAAAAAAAAAAAAAAA

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Fig. 1. Nucleotide sequence of pp38 protein cDNA. Two EcoRI sites are indicated in the boxes. The predicted amino acid sequence of the ORF is shown by the 3-letter code below the nucleotide sequence. The initiation codon and the termination codon of the longest ORF are underlined with double lines. The primer sequence for the primer extension analysis is underlined. The genomic sequences of the GA-strain and RA-strain different from the cDNA are aligned below the cDNA sequence. A deduced amino acid predicted from genomic sequence different from the cDNA is also aligned with the nucleotide sequence.

nucleotide chain termination method [19].

Plasmid pBP1 consists of a 0.9 kbp EcoRI fragment from the longest cDNA inserted into pBluescript SK+. The BamHI/HindIII fragment of pBP1 was inserted between the BamHI and HindIII sites in prokaryotic expression vector pQE9 (Qiagen Inc., Chatsworth, U.S.A.) to construct pQP9. After construction, pQP9 was transformed into *E. coli* M15/REP-4 [20]. Expression of fusion protein was induced with 2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C for 2 hr with shaking. Bacterial pellets from 20 ml of culture were resuspended in 1 ml of lysis buffer (7% 2-mercaptoethanol, 50 mM Tris, 1% SDS) and boiled. The centrifuged supernatant was directly applied to SDS-polyacrylamide (PAGE) and blotted to a polyvinylidene difluoride (PVDF) membrane. Recombinant pp38 on the membrane was detected with monoclonal antibody M21 [11]. Recombinant pp38 was purified by affinity chromatography with Ni-nitrilotriacetate immobilized by cross-linking agarose (Ni-agarose), according to the manufacturer's recommendations [9]. The recombinant fusion protein was eluted with 8 M urea, 0.1 M NaH₂PO₄, 20 mM 2-mercaptoethanol, 0.01 M Tris, pH 5.9. The protein concentration was measured with a Bio-Rad protein assay kit. Solubilization of the purified recombinant protein was performed with glutathione [7]. Purified protein in 8 M urea solution was stepwise (100 μ g/hr) added to 30 ml of refolding solution (0.1 M Tris-acetate buffer, pH 8.0, 1 mM reduced glutathione, 0.5 mM oxidized glutathione and 1 mM EDTA). The refolded products were concentrated in a dialysis bag.

RESULTS AND DISCUSSION

By plaque hybridization, 12 positive phages were obtained. In these phages, 4 clones contained the entire ORF and each of the other cDNAs contained only a part of the ORF. The DNA sequences of the longest two cDNAs were entirely matched and revealed that the cDNA insert consisted of 1739 bp with a 19-base poly(A) tail (Fig. 1). The 5' end of the cDNA was assigned to nt1. TATA sequence existed on nt-42. Cui *et al.* [6] predicted a transcription-starting site at nt-27. A primer extension analysis performed with an oligonucleotide primer (5'-GCTCTCGTCGGACGGGAGGCGGCGGTATAG-GATAAG-3') indicated that the 5' end of the mRNA for pp38 was 15 bp upstream of the 5' end of the cDNA sequence (data not shown). The primer extension analysis and sequencing of the cDNA indicated that the 870-nucleotide-long ORF was the largest ORF on the mRNA for pp38. Referred to Cui's data, 3 nucleotides for the cDNA and the genomic DNA were found to differ: a G in cDNA compared with an A in the genomic DNA at nt680, resulting in an arginine predicted from cDNA compared with a glutamine predicted from genomic DNA at amino acid 109; a T in cDNA compared with an A in the genomic DNA at nt1209 the third position of a codon within a predicted ORF, resulting in no predicted amino acid

Fig. 2. Western blots of proteins. a, lysate of strain CVI-988 MDV-infected CEF (CVI); b, lysate of pQP9-transformed M15 [REP4] with 2-hr induction; c, Ni-affinity-purified recombinant pp38. Molecular weight markers are indicated on the left side of the filter. Estimated molecular weights referring to the molecular weight markers are indicated on the right side of the filter.

change; and a C in cDNA compared with a T in the genomic DNA at nt1260, which was in the 3' noncoding region of a predicted ORF.

The entire 870-nucleotide-long ORF, except for the first ATG, was expressed as a recombinant protein by using the constructed plasmid pQP9. During construction of pQP9, a 17 bp BamHI/EcoRI vector (pBluescript) fragment and a 12 bp EcoRI/HindIII vector fragment were inserted at the junctions between the vector and viral sequence. As a result, the recombinant pp38 protein was expressed as a fusion protein with 16 amino acid residues (N-MRGSHHHHHGSPGLQ-C) on the N-terminus of the polypeptide except for the first ATG coded for pp38 cDNA. Because the 16 amino acid residues have affinity with Ni, this is called an affinity tag. The affinity tag resulted in an increase in molecular weight of 930 over the original protein. Recombinant protein was detected as a 39 kDa band on a blotted PVDF membrane. Four additional bands (38, 36, 34 and 24 kDa) were also observed on the membrane (Fig. 2, lane b). Ni-affinity the 39 kDa peptide was purified by Ni-affinity chromatography from the *E. coli* proteins (Fig. 2, lane c). The affinity and purity of the 39 kDa peptide was confirmed by means of Coomassie-stained SDS-PAGE gel. The 39 kDa protein was found as a strong band with a trace of 25 kDa *E. coli* protein (data not shown). The 38, 36, 34 and 24 kDa

M21-reacted bands were not found after the Ni-affinity chromatography (Fig. 2, lane c). These data indicated that the four bands had no affinity tag on the N-termini. When the *E. coli* was cultured without IPTG after induction, the 39 kDa peptide was decreased and the four smaller bands were increased (data not shown). This suggested that the 4 additional bands were the degradation products of the 39 kDa product. As a purified recombinant protein, 5 milligrams was obtained from 100 ml of culture. From this protein, 3 mg of solubilized protein was obtained after renaturation.

Based on the base sequence of the pp38 cDNA and the primer extension analysis, the 876 bp ORF was confirmed as the longest ORF in the pp38 mRNA (Fig. 1). In this report, the 39 kDa protein in pQP9-transformed *E. coli* suggested the use of this ORF in virus-infected cells (Fig. 2). The difference in mobility of recombinant pp38 and the pp38 in MDV infected cells could be explained by the molecular weight increase due to the affinity tag peptide.

Recombinant pp38 can be used in functional analysis for three reasons. First, it has almost the same mobility on PAGE gel as the native protein (Fig. 2A). Second, it can be used in a purified state. Third, an adequate proportion (c.a. 60%) of recombinant protein can be obtained in the soluble state. In conclusion, based on the confirmed 876 bp ORF, soluble recombinant pp38 was purified as a tool for functional tests of pp38 protein.

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