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Proteolytic processing of poliovirus polypeptides: Antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs

(proteinase/*in vitro* translation/immunoprecipitation/genome map)

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ABSTRACT **Proteolytic processing of poliovirus polypeptides was examined by the addition of antibodies directed against the viral proteins P3-7c and P2-X to a cell-free translation extract prepared from infected HeLa cells. Antisera to P3-7c specifically inhibited *in vitro* processing at Gln-Gly pairs. Partial amino acid sequence analysis revealed a second Tyr-Gly pair that is utilized in protein processing. Neither Tyr-Gly cleavage is affected by antibody to P3-7c. Anti-P3-7c antibodies react not only with P3-7c but also with P3-6a and P3-2, two viral polypeptides NH_2-coterminal with P3-7c. Preimmune and anti-P2-X antibodies had no effect on the processing of poliovirus proteins *in vitro*. We conclude that the activity responsible for processing poliovirus polypeptides at Gln-Gly pairs resides in the primary structure of P3-7c (or P3-2 and P3-6a) and not in P2-X.**

Picornaviruses such as poliovirus produce their proteins in an unusual way. Virus-specific mRNA, which differs from genome RNA only at its 5′ terminus (1–3), is translated to produce one large polyprotein that is subsequently processed by proteolytic cleavage into smaller, functional proteins (4–7). The nucleotide sequence of the poliovirus genome has a long reading frame encompassing 89% of the RNA (8, 9), an observation in accordance with polyprotein synthesis. From partial sequence analyses of poliovirus-specific proteins, a complete genetic map has been constructed that reveals not only the complete amino acid sequences of individual polypeptides but also the cleavage sites utilized for proteolytic processing (10–14). Cleavage was found to occur between glutamine and glycine at nine processing sites within the polyprotein; these sites occur in all three regions (P1, P2, P3; Fig. 1). Tyr-Gly and Asn-Ser are also utilized as processing signals but these amino acid pairs are less common.

A major unsolved question of poliovirus replication is the origin and the specificity of one or more proteinases involved in polypeptide processing. The available evidence shows that picornaviruses code for at least one proteinase (16). Korant *et al.* (17) suggested that polypeptide P2-X of poliovirus (Fig. 1) is a proteinase. In the case of the closely related encephalomyocarditis (EMC) virus, a smaller polypeptide (p22) mapping in the replicase region (P3) of the genome (corresponding to P3-7c of poliovirus) has been shown to be associated with proteolytic cleavage of proteins from both the P1 and P3 regions (18, 19). Because only limited sequence information is available for the RNA and proteins of EMC virus, the cleavage sites within its polyprotein cannot yet be identified. However, it is known that a single Gln-Gly pair is cleaved in the capsid (P1) region of mengovirus (20), a virus closely related to EMC virus.

In this paper we describe the use of monospecific antisera prepared against poliovirus proteins P2-X and P3-7c to examine processing that occurs at Gln-Gly and Tyr-Gly cleavage sites in a poliovirus-specific cell-free translation system. These studies have allowed us to conclude that the amino acid sequence of P3-7c is involved in polyprotein processing at Gln-Gly cleavage sites. Our results show that at least one more proteinase, possibly of host cell origin, is required for complete polyprotein processing.

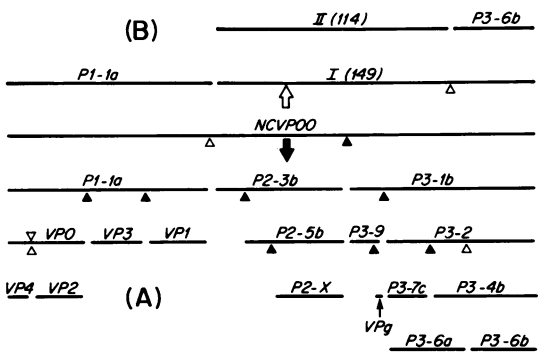

FIG. 1. Cleavage of poliovirus proteins in the absence (*A*) and presence (*B*) of anti-P3-7c IgG (8, 10, 15). Protein molecules are represented by a solid line. ▲, Cleavages at Gln-Gly pairs; △, cleavages at Tyr-Gly pairs; ⧖, cleavage at an Asn-Ser pair. If cleavage at Gln-Gly pairs is inhibited by anti-P3-7c IgG, polypeptide I would contain the amino acid sequence of P2-3b and P3-1b and polypeptide II would contain the amino acid sequence of P2-3b, P3-9, and P3-6a. Numbers in parentheses are molecular weights $\times 10^{-3}$.

MATERIALS AND METHODS

Preparation of Infected Cell Extracts. Extracts were prepared from suspension cultures of S3 HeLa cells (5×10^6 cells per ml) infected with poliovirus type 1 (Mahoney) as described (10). Harvested cells were allowed to swell in hypotonic buffer (10 mM NaCl/10 mM Tris·HCl, pH 7.4/1.5 mM $MgCl_2$) for 15 min at 0°C and then homogenized (Dounce). The cellular extract was subjected to fractionation by differential centrifugation for 30 min at 20,000 × *g*. The membrane pellet recovered after centrifugation was solubilized in TGKD buffer [15 mM Tris·HCl, pH 7.5/25% (vol/vol) glycerol/10 mM KOAc/10 mM dithiothreitol] containing 0.5% Nonidet P-40 and homogenized gently. The homogenate was incubated for 30 min at 0°C and centrifuged again for 30 min at 20,000 × *g*.

Preparation of Viral Polypeptides and Immunization of Rabbits. The membrane-enriched supernatant (from above) was applied to a phosphocellulose column equilibrated with TGKD buffer/0.5% Nonidet P-40. Poliovirus proteins bound

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Abbreviations: EMC virus, encephalomyocarditis virus; DBM, diazobenyloxymethyl.

to the resin were eluted with a continuous gradient (10–400 mM) of KOAc at pH 7.5. Peak fractions were identified by $NaDodSO_4$/12.5% polyacrylamide gel electrophoresis and autoradiography (21). Fractions containing P2-X and P3-7c were pooled, acetone precipitated, and subjected to preparative $NaDodSO_4$/gradient (10–18%) polyacrylamide gel (25 × 15 × 1.3 cm) electrophoresis. Gel-purified proteins (P2-X or P3-7c) were eluted as described (10) except that carrier protein was not included during the elution. Rabbit antisera were raised to P2-X or P3-7c by a primary injection of approximately 100 μg (total protein) in elution buffer into the popliteal lymph node of New Zealand White rabbits. Intramuscular booster injections of approximately 50 μg (total protein) in incomplete Freund's adjuvant were given once a week for 4 weeks.

Immunoprecipitation, Electrophoretic Transfer of Poliovirus Proteins, and Immunoautoradiography. Immunoprecipitation of proteins from extracts of radiolabeled, infected cells and of proteins eluted from gels was performed as described (13). Precipitated proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography (22).

Sera from immunized rabbits were also screened for reactivity with poliovirus proteins and HeLa cell proteins by the technique of electrophoretic transfer to diazobenzyloxymethyl-paper (DBM-paper) and immunoautoradiographic analysis. Extracts of unlabeled infected and uninfected cells and of [^{35}S]methionine-labeled infected cells were prepared as described (10). DBM-paper was prepared according to Alwine *et al.* (23). Electrophoretic transfer of proteins from $NaDodSO_4$/12.5% polyacrylamide gels to paper was carried out as described (24). After transfer, the DBM-paper was incubated first with rabbit antiserum diluted 1:20, and then with ^{125}I-labeled protein A from *Staphylococcus aureus* (kindly provided by Wes Yonemoto) according to the method of Renart *et al.* (25). The paper was dried and autoradiographed.

Purification and Concentration of IgG. Rabbit antisera were dialyzed against 20 mM Tris·HCl, pH 8.0/28 mM NaCl overnight at 4°C. Samples (5 ml) were then applied to a column of DEAE AFFI-gel Blue (Bio-Rad) equilibrated with the above buffer. Unbound protein fractions containing the immunoglobulins were washed through the column with three bed volumes of the same buffer. Fractions were analyzed for absorbance at 280 nm. Peak fractions were pooled and the proteins were concentrated by a 50% ammonium sulfate precipitation step. The resulting pellet was dissolved in 0.5 ml 10 mM Tris·HCl, pH 8.0/10 mM KCl and dialyzed overnight at 4°C against the same buffer. The final concentration of protein (approximately 10 mg/ml for each IgG preparation) was determined by the method of Lowry *et al.* (26).

Amino Acid Sequence Analysis. Radioactively labeled P3-6a and P3-6b that had been polyacrylamide gel-purified were subjected to automated Edman degradation in a Beckman 890C sequencer as described (10, 11, 27). The coding region compatible with the amino acid sequence data was then located within the nucleotide sequence of poliovirus RNA (8, 9).

Cell-Free Protein Synthesis. The cell-free translation system was prepared from an extract of poliovirus-infected HeLa cells 3 hr after infection essentially as described (28). The standard incubation mixtures (61 μl) contained 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 0.2 mg of creatine kinase per ml, 28 mM Hepes (pH 7.4 with KOH), 2.2 mM $Mg(OAc)_2$, 90 mM KCl, 1 mM dithiothreitol, 19 unlabeled amino acids at 19 μM each, 50 μg of tRNA per ml, and 13.5 μl of infected HeLa cell extract. Rabbit IgG fractions, purified as described above, were added to separate reaction mixtures. Samples were precipitated with trichloroacetic acid and subjected to $NaDodSO_4$/gradient (10–18%) polyacrylamide gel electrophoresis.

RESULTS

Production and Screening of Antisera. Poliovirus nonstructural proteins P2-X and P3-7c were purified from membrane-enriched fractions of poliovirus-infected HeLa cells by phosphocellulose chromatography and $NaDodSO_4$/polyacrylamide gel electrophoresis. These radiochemically pure proteins were used to immunize rabbits. Antisera obtained from immunized rabbits were used to immunoprecipitate [^{35}S]methionine-labeled poliovirus proteins (Fig. 2). Anti-P2-X antiserum obtained from each of two animals was able to precipitate purified labeled P2-X (lanes 2 and 3). Labeled P3-7c, purified as described above, was immunoprecipitated by antiserum from a rabbit that had been injected with P3-7c (lane 5). Preimmune serum did not precipitate P2-X or P3-7c (lanes 4 and 6, respectively).

We observed considerable immunoprecipitation of heterologous polypeptides from a lysate of [^{35}S]methionine-labeled infected cells with both anti-P2-X and anti-P3-7c sera (Fig. 2, lanes 7–9). The precipitation of unrelated polypeptides by specific poliovirus antisera has been observed previously (13) and may be due to coprecipitation of poliovirus protein aggregates in radiolabeled extracts. Therefore, immunoautoradiographic analysis (24, 25) was used to determine which poliovirus polypeptides react with anti-P2-X and anti-P3-7c antisera. [^{35}S]-Methionine-labeled poliovirus proteins ranging in molecular weight from 20,000 to 97,000 were transferred to DBM-paper (Fig. 3). Anti-P3-7c antiserum reacted with viral proteins P3-2, P3-6a, and P3-7c. This result is consistent with reported data

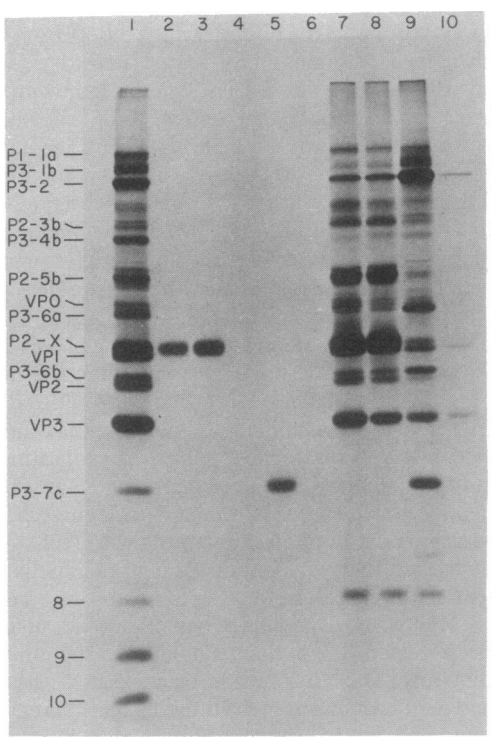

FIG. 2. Autoradiograph of a 12.5% $NaDodSO_4$/polyacrylamide gel of immunoprecipitated poliovirus polypeptides. Purified [^{35}S]methionine-labeled P2-X was incubated with anti-P2-X antisera from two different rabbits (lanes 2 and 3) and normal rabbit serum (lane 4). Purified [^{35}S]methionine-labeled P3-7c was incubated with anti-P3-7c antiserum and normal rabbit serum (lanes 5 and 6, respectively). Infected HeLa cell lysates labeled with [^{35}S]methionine were immunoprecipitated with 20 μl of anti-P2-X antisera (lane 7, rabbit A; lane 8, rabbit B), 20 μl of anti-P3-7c antiserum (lane 9), or 20 μl of normal rabbit serum (lane 10). Lane 1 represents an extract of [^{35}S]methionine-labeled infected HeLa cells not immunoprecipitated.

(10) and with protein sequence data reported in this communication (see below). A weak reaction of anti-P3-7c antiserum with proteins having approximate molecular weights of 52,000 and 68,000 also was observed in the lanes containing extracts of both infected and uninfected HeLa cells. This result suggests that these may be cellular proteins that crossreact slightly with anti-P3-7c antiserum. Anti-P2-X antiserum reacted with three poliovirus polypeptides, P2-3b, P2-5b, and P2-X, in a transfer of a virus-infected cell extract. These proteins all share amino acid sequences (11, 15). No visible reaction was observed with proteins transferred from an uninfected HeLa cell extract. Preimmune serum did not react with any proteins transferred from lysates of infected or uninfected HeLa cells (data not shown).

Amino Acid Sequence Analysis of P3-6a and P3-6b. Rueckert *et al.* (15) have suggested that polypeptides P3-6a and P3-6b may be alternate cleavage products of protein P3-2 (Fig. 1). Because P3-6a (but not P3-6b) reacted with anti-P3-7c antiserum (Fig. 3, lane B), we determined the precise map positions of P3-6a and P3-6b by following the strategy used previously: partial amino acid sequence analysis and alignment of the predicted with the experimental sequences (8, 10, 11, 12). Fig. 4 shows profiles of automated Edman degradations of radiolabeled, gel-purified P3-6a and P3-6b. P3-6a has the same amino terminus as P3-2 and P3-7c (10). The data for P3-6b place its amino terminus at the glycine residue encoded by nucleotides 6,423–6,425 according to the numbering system of Kitamura *et al.* (8). The amino acid pair cleaved in P3-2 to generate P3-6a and P3-6b is Tyr-Gly. This Tyr-Gly is only the second Tyr-Gly cleavage site we have identified within the amino acid sequence of the poliovirus polyprotein (11). Size considerations suggest that the carboxy terminus of P3-6b is coincident with the

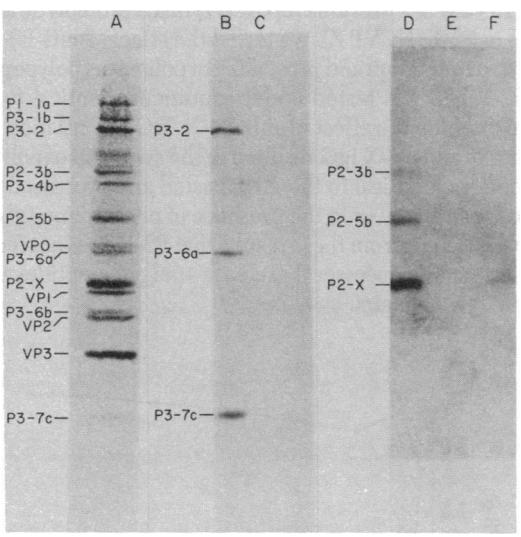

FIG. 3. Immunoautoradiographic detection of poliovirus proteins transferred to DBM-paper. A lysate of [^{35}S]methionine-labeled infected HeLa cells was electrophoretically transferred from a 12.5% NaDodSO$_4$/polyacrylamide gel to DBM-paper and the autoradiograph was exposed for 18 hr to produce a marker lane (lane A). A lysate of unlabeled poliovirus-infected HeLa cells (lane B) and a lysate of unlabeled mock-infected HeLa cells (lane C) were transferred to DBM-paper and treated with anti-P3-7c antiserum and then ^{125}I-labeled protein A. Lanes D and E, representing autoradiographs of the same protein blot "erased" with urea and 2-mercaptoethanol and then treated with anti-P2-X antiserum and ^{125}I-labeled protein A, show the reaction of anti-P2-X antiserum with the lysates of infected and uninfected HeLa cells, respectively. [^{3}H]Glycine-labeled P2-X was purified, transferred to DBM-paper (lane F), and treated with anti-P2-X antiserum and ^{125}I-protein A.

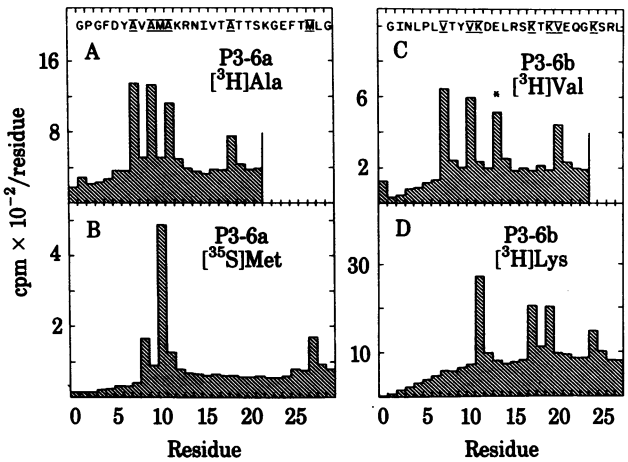

FIG. 4. Radiochemical amino-terminal sequence analysis of poliovirus polyprotein cleavage products P3-6a and P3-6b. NaDodSO$_4$/polyacrylamide gel purified P3-6a (*A* and *B*) and P3-6b (*C* and *D*) labeled with [^{3}H]alanine (*A*), [^{35}S]methionine (*B*), [^{3}H]valine (*C*), or [^{3}H]lysine (*D*) were prepared and subjected to amino-terminal sequence analysis using a Beckman 890C automated sequencer as described (10). The total radioactivity obtained in the amino acid residue from each Edman degradation cycle is plotted versus the cycle number. In *A* and *C* are given (in single-letter amino acid code) the amino acid sequence corresponding to the protein as predicted from the nucleotide sequence of poliovirus RNA (8, 9). The peak of radioactivity at residue 13 (indicated by *) is the result of contamination of our P3-6b sample with viral protein VP2 (12). The amount of radioactivity applied to the sequencer for each sample was: *A*, 41,100 cpm; *B*, 9,900 cpm; *C*, 32,000 cpm; and *D*, 83,800 cpm.

terminus of the polyprotein. Therefore, protein P3-6b can be produced by a single cleavage and is a specific indicator of the utilization of Tyr-Gly as a site for proteolytic processing. We have made use of this observation to test the specificity of inhibition of cleavage by antibodies to protein P3-7c (see below).

Effect of Anti-P3-7c IgG on Poliovirus Protein Processing *in Vitro*. Previous studies have shown that picornavirus proteins can be faithfully synthesized and processed *in vitro* in a rabbit reticulocyte lysate (29–31). However, the efficiency of translation of exogenous poliovirus RNA is poor when compared to that of EMC virus RNA (ref. 31; unpublished data). We therefore made use of the endogenous *in vitro* translation system described by Celma and Ehrenfeld (28). This system is prepared from extracts of poliovirus-infected HeLa cells and appears to translate and process viral proteins that are nearly identical to those observed in infected HeLa cells *in vivo* (compare lanes M in Fig. 5 *A* and *B*) (32). To determine whether the poliovirus-encoded polypeptides P2-X or P3-7c are involved in cleavage events that produce the structural and nonstructural polypeptides of poliovirus, a series of assays was performed in which increasing concentrations of purified anti-P3-7c, anti-P2-X, or preimmune IgG were added to *in vitro* translation mixtures containing a fixed concentration of protein.

When the translation mixtures and anti-P3-7c IgG were incubated together prior to translation, the pattern of synthesized proteins changed drastically (Fig. 5*A*). There was a decrease in the degree of proteolytic processing during *in vitro* translation as the concentration of anti-P3-7c IgG increased. The poliovirus proteins produced by cleavages at Gln-Gly pairs became less prominent with increasing antibody concentration, and there was a corresponding accumulation of large precursor polypeptides. More importantly, the inhibition, by anti-P3-7c, of cleavage was specific for Gln-Gly pairs and did not affect cleavage at Tyr-Gly sites (see, for example, P3-2, P3-6a, P2-X, and VP3). The production of protein P3-6b was unaffected by anti-P3-7c

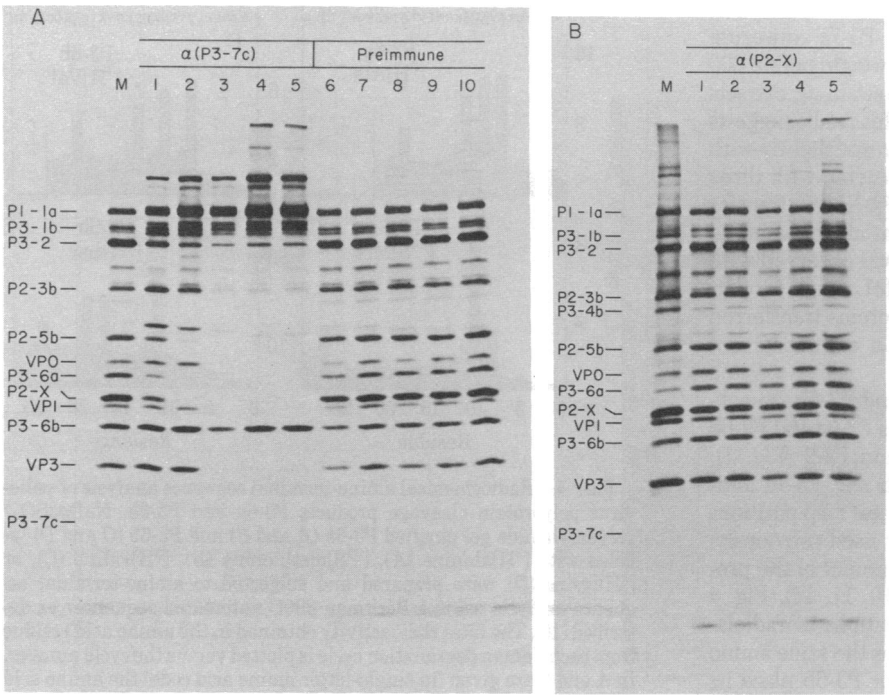

FIG. 5. Effect of anti-P2-X and anti-P3-7c on the processing of poliovirus proteins *in vitro*. Translation reaction mixtures were preincubated on ice for 1 hr in the presence of anti-P3-7c, anti-P2-X, or preimmune IgG or in the absence of IgG. [^{35}S]Methionine was added (246 μCi/ml) and the samples were incubated for 17 hr at 30°C. Aliquots (10 μl) were withdrawn from each reaction and analyzed. (*A*) Effect of adding increasing amounts of anti-P3-7c IgG—lane 1, 15 μg; lane 2, 50 μg; lane 3, 150 μg; lane 4, 250 μg; lane 5, 300 μg—or preimmune IgG—lane 6, 15 μg; lane 7, 50 μg; lane 8, 150 μg; lane 9, 250 μg; lane 10, 300 μg. Poliovirus proteins synthesized in an extract of infected HeLa cells in the absence of antisera are displayed in lane M. (*B*) Effect of adding increasing amounts of anti-P2-X IgG—lane 1, 15 μg; lane 2, 50 μg; lane 3, 150 μg; lane 4, 250 μg; lane 5, 300 μg—to the translation reaction. Lane M displays an *in vivo* lysate from poliovirus-infected HeLa cells. Note that in this experiment P3-7c is barely detectable. We have observed increased production of P3-7c during *in vitro* translation with other extracts of infected HeLa cells.

IgG at any concentration. Similarly, P1-1a also remained stable in the presence of anti-P3-7c, although this effect was obscured somewhat by the accumulation of large polypeptides. Thus, the activity responsible for protein processing at a Tyr-Gly pair is not inhibited by the IgG preparation that inhibits processing at all known Gln-Gly cleavage sites.

The amino terminus of P2-3b is also produced by cleavage of a Tyr-Gly within the polyprotein whereas its carboxy terminus is produced by cleavage of a Gln-Gly (Fig. 1) (10, 11, 14). Because processing of the authentic poliovirus proteins is inhibited by anti-P3-7c, it is likely that the observed protein species with molecular weights greater than 97,000 (the molecular weight of the largest "primary" cleavage product of the polyprotein, P1-1a) represent uncleaved precursor polypeptides having the amino terminus of P2-3b. Fig. 1*B* shows how two such polypeptides with apparent molecular weights of 150,000 and 114,000 might be produced. Accordingly, the observed increase in these precursor molecules with increasing amounts of anti-P3-7c (Fig. 5*A*) can be explained if processing occurs at Gln-Gly pairs to a lesser extent.

To establish that the inhibition of proteolysis resulted from the inactivation of a proteinase or a proteinase cofactor by anti-P3-7c IgG, translation in the presence of preimmune IgG was tested under identical conditions (Fig. 5*A*). The poliovirus polypeptides synthesized were identical to those synthesized and processed in the absence of normal rabbit IgG. These results show that there is no appreciable nonspecific proteinase activity in our rabbit IgG and that translation of poliovirus polypeptides proceeds unhindered by the addition of preimmune rabbit IgG.

In order to quantitate the extent of inhibition of cleavage in the presence of anti-P3-7c IgG, microdensitometer tracings were taken of the gel patterns shown in Fig. 5*A*. The production of P2-X [produced by cleavage at two Gln-Gly pairs (11) within P2-3b] relative to the production of P3-6b (cleavage at one Tyr-Gly pair) was then measured at the different concentrations of IgG used in each *in vitro* translation incubation mixture. The relative synthesis of P2-X and P3-6b varied only slightly with increasing concentrations of preimmune IgG (Fig. 6). However, the ratio of P2-X to P3-6b decreased sharply in the presence of increasing concentrations of anti-P3-7c IgG and leveled off at an IgG concentration at which no radiochemically detectable P2-X was produced. These data illustrate the pronounced and specific effect of antibody directed against P3-7c on proteolytic cleavage at Gln-Gly pairs.

Effect of Anti-P2-X IgG on Poliovirus Protein Processing *in Vitro*. In view of a previous report (17) that a proteolytic activity resides in P2-X (NCVP X), we tested the effect of anti-P2-X IgG on *in vitro* translation and processing of poliovirus polypeptides. Anti-P2-X IgG was tested under conditions identical to those used to examine the effect of anti-P3-7c IgG. Translation in the presence of anti-P2-X IgG resulted in the synthesis of poliovirus polypeptides identical to those translated and processed in the absence of antibody or in the presence of preimmune IgG (Fig. 5*B*). We conclude from these results that P2-X does not function in the enzymatic processes that cleave viral polypeptides at Gln-Gly and Tyr-Gly pairs.

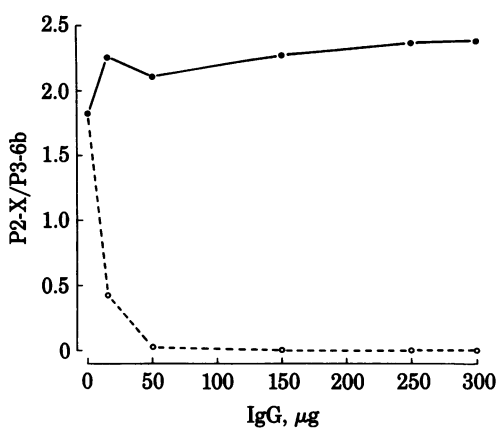

FIG. 6. Effect of IgG concentration on the relative amounts of P2-X and P3-6b synthesized *in vitro*, based on microdensitometer tracings of the autoradiogram shown in Fig. 5*A*. The ratio of P2-X to P3-6b in the presence of preimmune IgG (●) or anti-P3-7c IgG (○) was determined for each concentration of antibody.

DISCUSSION

Several studies have provided evidence that picornaviruses code for at least one proteinase. The most convincing experiments are those in which a proteinase activity was synthesized *in vitro* under the direction of EMC virus RNA (29, 31) and was partially purified from the EMC virus proteins synthesized in the reticulocyte lysate (18). Independently, a similar activity capable of cleaving the EMC virus precursor polypeptide was partially purified from EMC virus-infected ascites cells (19). The proteolytic activity of EMC virus is associated with polypeptide p22, corresponding to P3-7c of poliovirus.

Our approach to the study of poliovirus protein processing has utilized inhibition of proteinase activity by monospecific antibodies added to an endogenous *in vitro* translation system (28). These antibodies were raised against viral proteins P2-X and P3-7c. The results presented in this paper lead us to conclude that the amino acid sequence of P3-7c, rather than P2-X, plays an essential role in the proteolytic processing of poliovirus polypeptides at specific Gln-Gly pairs. P3-7c itself may be a proteinase. Alternatively, the proteolytic activity may reside in other virus-specific polypeptides that contain the P3-7c amino acid sequence (i.e., P3-6a and P3-2). Complete purification and characterization of this activity will distinguish between these possibilities.

The finding that anti-P2-X has no effect on processing of poliovirus polypeptides *in vitro* suggests that P2-X is not involved in cleavages at Gln-Gly or Tyr-Gly sites. This conclusion is at variance with a previous report (17). However, the proteolytic activity that was observed to be associated with P2-X may have been due to copurification of P3-6a and P2-X. Although we have no evidence, we consider it possible that P3-6a may also possess proteinase activity.

The origin of the enzyme(s) responsible for cleavage at Tyr-Gly sites remains to be determined. It is possible that either a second viral polypeptide or a cellular proteinase processes viral proteins at specific Tyr-Gly pairs. If a cellular enzyme is responsible, it could cleave the polyprotein during the earliest stages of infection (before P3-7c has been formed) into P1-1a, polypeptide II, and P3-6b (Fig. 1*B*). Subsequent cleavages of polypeptide II—either by the *trans* action of one polypeptide II on another or by autocatalytic cleavage of this protein at specific Gln-Gly pairs—would then produce the viral proteinase. Experiments suggesting autocatalytic cleavage of EMC virus protein D (corresponding to poliovirus protein P3-2) have been reported recently (33).

At least 9 of the 13 Gln-Gly pairs and 2 of the 10 Tyr-Gly pairs found within the polyprotein are utilized in processing (8, 10–12, 14). The parameters controlling cleavage specificity (other than cleaved amino acid pairs) and the sequence of proteolytic events are not well understood. It is likely that the ordering of processing events is a consequence of and responsible for the accessibility of specific cleavage determinants for the proteinases. The initial ("primary") cleavages in the P2–P3 regions of the polyprotein may be important determinants of the processing pathway that is then followed.

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