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Relationship of Eukaryotic Initiation Factor 3 to Poliovirus-Induced p220 Cleavage Activity

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The cleavage of the p220 subunit of eukaryotic initiation factor 4F (eIF-4F) that is induced by the poliovirus protease 2A has been shown previously to require another translation initiation factor, eIF-3. The role of eIF-3 in this cleavage reaction, however, is not known. An antiserum was raised against human eIF-3 and used to analyze the eIF-3 subunit composition in poliovirus-infected and uninfected HeLa cells and after incubation of eIF-3 in vitro with viral 2A protease. No evidence for $2A^{pro}$-dependent cleavage of any eIF-3 subunit was detected. Infected cells contain an activity that catalyzes the cleavage of p220 to a specific set of cleavage products. This activity is thought to be an activated form of a latent cellular protease. The p220-specific cleavage activity was partially purified. It was resolved from eIF-3 by both gel filtration and anion-exchange chromatography. Neither intact eIF-3 nor any detectable subunits of eIF-3 were found to copurify with the p220-specific cleavage activity. The latter activity behaves as a protein of 55,000 to 60,000 molecular weight and is inhibited by alkylating agents and metals, which indicates the presence of essential thiol groups. When this activity was incubated with partially purified p220, cleavage occurred only in the presence of eIF-3. Thus, eIF-3 appears to play a role in the p220 cleavage cascade which is subsequent to the $2A^{pro}$-induced activation of the p220-specific protease.

Infection of HeLa cells with poliovirus results in rapid inhibition of host cell protein synthesis (for reviews, see references 11 and 41). The inability to translate cellular mRNAs was shown to result from a defect in the activities of translation initiation factors (18, 22). Eukaryotic initiation factor 4F (eIF-4F) was able to restore translation of capped mRNAs in extracts from poliovirus-infected cells, implying that this was the defective factor in these cells (13, 45, 46). This view was supported by the observation that the γ or p220 subunit of eIF-4F is specifically cleaved in poliovirus-infected cells (14) and that the early shutoff of host cell protein synthesis correlates well with the loss of intact p220 (14, 30). Alterations in the structure and activities of other initiation factors have not been detected (6, 9, 14, 19, 27).

eIF-4F is composed of three polypeptide subunits. The α subunit, also known as the cap-binding protein or eIF-4E, is a 25,000-molecular-weight protein which can be cross-linked to oxidized reovirus mRNA caps in the absence of ATP and magnesium (43). This is believed to be the major protein responsible for the specific recognition of capped mRNAs by the translational apparatus. The β subunit, also known as eIF-4A, is an ATP-dependent RNA helicase which removes secondary structures from the 5′ end of mRNAs (10, 16, 25, 36, 38). The γ subunit, p220, is visualized on sodium dodecyl sulfate (SDS)-polyacrylamide gels as a family of at least four bands with an average molecular weight of about 220,000. The contribution of p220 to eIF-4F function is not known, but it appears to be required for the full activity of this factor (13, 45). The exact mechanism by which eIF-4F stimulates translation of capped mRNAs is unclear. Proposed activities include promoting the helicase function of eIF-4A, perhaps by bringing it into association with the 5′ end of the mRNA (37, 38, 42). It has also been proposed that an eIF-4F–eIF-3 interaction may promote binding of the mRNA to an eIF-3–ribosome complex (15, 17, 46).

In extracts from poliovirus-infected cells, intact p220 is not detected, but new antigenically related polypeptides are visualized with molecular weights ranging from 100,000 to 130,000. Extracts from poliovirus-infected cells contain an activity which catalyzes the cleavage of p220 to these cleavage products (14). This activity is distinct from either of the poliovirus-encoded proteases, $3C^{pro}$ (26, 28) or $2A^{pro}$ (31). Several lines of evidence indicate that although $2A^{pro}$ does not itself catalyze the cleavage of p220, expression of active $2A^{pro}$ is required for p220 cleavage (3, 24, 29, 35, 44). The current model is that $2A^{pro}$ activates a latent cellular protease activity which then cleaves p220. This model in its simplest form is presented schematically in Fig. 1. Although this is the currently accepted working model for p220 cleavage, other models that are consistent with available data can also be constructed. The mechanism by which $2A^{pro}$ activates this p220-specific cleavage activity is unknown, but two recent reports provide direct evidence that it is the protease activity of $2A^{pro}$ that is required for the induction of p220 cleavage (20, 50). This activated p220-specific protease activity has been partially purified from poliovirus-infected cells, but has never been identified (28).

We recently showed that eukaryotic initiation factor 3 (eIF-3) is required for $2A^{pro}$-dependent cleavage of p220 in vitro; however, the position of eIF-3 in the proposed protease cascade is unknown (48). It could itself be the latent form of the p220-specific protease, or alternatively, it could in some way facilitate either $2A^{pro}$-dependent formation of the active p220-specific protease or the p220 cleavage reaction. In this report, we compare eIF-3 in poliovirus-infected and uninfected cell extracts and examine its relationship to the

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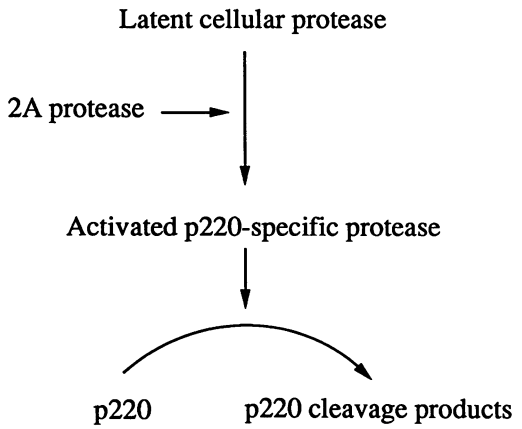

FIG. 1. Model for poliovirus-induced cleavage of p220. The current model for the induction of p220 cleavage by poliovirus $2A^{pro}$ is shown. The poliovirus $2A^{pro}$ is thought to activate a latent cellular protease to an activated form capable of cleaving p220 to a set of immunologically related cleavage products.

p220-specific cleavage activity. We also show that the eIF-3 complex and the p220-specific cleavage activity separate during purification and that both proteins are required for efficient cleavage of p220.

MATERIALS AND METHODS

Materials. Antipain, leupeptin, pepstatin, aprotinin, and *N*-[*N*-L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E64) were purchased from Boehringer Mannheim Biochemicals, and *N*-ethylmaleimide, iodoacetamide, and phenylmethylsulfonyl fluoride were purchased from Sigma. Cbz-ValPheH (32) was the gift of Martin Rechsteiner.

Preparation of rabbit antiserum to eIF-3. eIF-3 purified from HeLa cells was the gift of John Hershey. Approximately 100 μg of protein was used to immunize a rabbit which then received three 50-μg booster injections. The

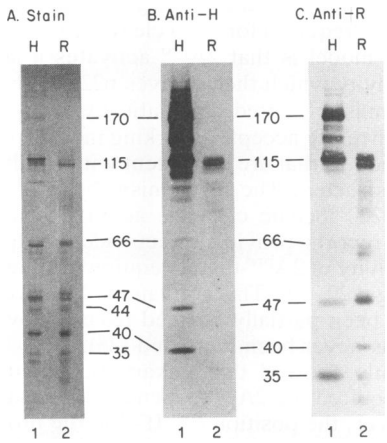

FIG. 2. Characterization of antiserum to human eIF-3. eIF-3 prepared from either HeLa cells (lane 1) or rabbit reticulocytes (lane 2) was analyzed on SDS-7.5% polyacrylamide gels. Proteins were visualized either by silver staining (A) or in immunoblots with antiserum raised to either human eIF-3 (B) or rabbit reticulocyte eIF-3 (33) (C).

polypeptide composition of the factor used to raise this serum is shown in Fig. 2A.

Poliovirus infection and preparation of cellular extracts. HeLa S3 cells were grown in suspension culture in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cells were infected with the Mahoney strain of poliovirus type 1 at a multiplicity of infection of 100 PFU per cell as previously described (7). To visualize proteins present in the infected extracts, 1.5 ml of infected cells were removed, and cell pellets were recovered by centrifugation for 30 s in a microcentrifuge and resuspended in 0.1 ml of RSB (10 mM Tris-Cl [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$). To cause cell lysis, 10 μl of 10% (vol/vol) Nonidet P-40 was added, and samples were shaken gently at 37°C for 2 min. Nuclei were removed by centrifugation for 1 min in a microcentrifuge, and the supernatant was mixed with SDS gel sample buffer and boiled for 3 min.

To prepare active proteins, either uninfected or poliovirus-infected HeLa cells were concentrated and disrupted in a Dounce homogenizer as previously described (4). Subcellular fractionation to obtain postmitochondrial and postribosomal supernatants and the ribosomal salt wash (RSW) were also performed as described previously (4). Proteins were precipitated from the RSW with 40% saturated ammonium sulfate and dialyzed against H buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4], 1.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.02% NaN_3) plus 100 mM KCl to give the RSW-A cut.

Column chromatography of extracts from infected and uninfected HeLa cells. Postribosomal supernatants were adjusted to 100 mM KCl columns were loaded, and dialyzed RSW-A cuts were loaded directly onto the columns. Anion-exchange chromatography was performed on a Mono-Q 5/5 prepacked column (Pharmacia) which had been equilibrated in H buffer plus 100 mM KCl. Bound proteins were eluted with a 100 to 500 mM KCl gradient. Gel filtration was performed on a Superose-6 HR 10/30 column (Pharmacia) equilibrated in H buffer plus 100 mM KCl. The exclusion volume was determined by the elution of blue dextran. The column was calibrated with thyroglobulin (669,000), apoferritin (443,000), β-amylase (200,000), bovine serum albumin (66,000), and ovalbumin (45,000).

Preparation of partially purified p220 substrate. A postribosomal supernatant was prepared from uninfected HeLa cells as previously described (4). The KCl concentration was adjusted to 100 mM, and the extract was loaded on a 12-ml P-11 phosphocellulose column (Whatman) which had been equilibrated in H buffer plus 100 mM KCl. Bound protein was eluted in a 100 to 400 mM KCl gradient. p220 eluted between 240 and 320 mM KCl. These fractions were pooled and adjusted to 1 mM $CaCl_2$. Pooled fractions were loaded on a 2-ml calmodulin Sepharose column (Pharmacia) equilibrated with 20 mM HEPES (pH 7.4)–1.5 mM $MgCl_2$–100 mM KCl–0.02% NaN_3–1 mM $CaCl_2$. p220 was bound to the column and was eluted in 20 mM HEPES (pH 7.4)–1.5 mM $MgCl_2$–100 mM KCl–0.02% NaN_3–5 mM EGTA.

Assay for p220 cleavage activity. Several types of assays for p220 cleavage were performed. Assays in which p220 cleavage is induced by $2A^{pro}$ expressed in *Escherichia coli* were performed as previously described (48). To assay the p220-specific cleavage activity in poliovirus-infected cell extracts, each 10-μl reaction mixture contained 2 μl of postmitochondrial supernatant from uninfected HeLa cells, 20 mM HEPES (pH 7.4), 10 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 1 to 5 μl of either an extract from poliovirus-infected HeLa cells or a column fraction derived

from the extract as indicated. In some experiments, the p220 substrate had been partially purified as described above. Each of these 10-μl reaction mixtures contained 3 μl of p220 substrate, 20 mM HEPES (pH 7.4), 40 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 3 μl of Mono-Q fraction containing p220-specific cleavage activity. Some of these reaction mixtures contained 500 ng of purified HeLa eIF-3.

RESULTS

Analysis of eIF-3 from uninfected and infected HeLa cells. The observation that eIF-3 is required for p220 cleavage suggests that it could be the latent form of the p220-specific cleavage activity. If activation of the latent enzyme by $2A^{pro}$ is a proteolytic event, then it is predicted that one or more of the eIF-3 subunits should be a substrate for cleavage by $2A^{pro}$. Previous experiments had not detected any changes in eIF-3 subunits from infected cells in immunoblots with a serum raised to rabbit reticulocyte eIF-3 (14). Not all eIF-3 subunits were reactive with that serum, and some regions of the gel were difficult to visualize because of reactivity of the serum with p220 and its cleavage products. An antiserum against human eIF-3 was recently raised in our laboratory, and experiments were performed to determine whether any changes in eIF-3 in poliovirus-infected cells could be detected with this serum. The characterization of this antiserum is shown in Fig. 2. eIF-3 is a large complex consisting of at least eight antigenically distinct subunits (21, 34, 39). Most of these subunits are visualized in the stained gel of purified eIF-3 (Fig. 2A), except that in the rabbit reticulocyte eIF-3, p170 is present in a cleaved form (20a, 48). The serum raised to HeLa eIF-3 recognized several eIF-3 subunits including p170, p115, p47, p40, and, more weakly, p66 (Fig. 2B). The serum reacted with fewer subunits in the purified rabbit reticulocyte eIF-3 (Fig. 2B, lane 2). The main immunoreactive band has a molecular weight of about 115,000 (Fig. 1B), but immunoblots of rabbit reticulocyte lysates indicate that this is likely to be a proteolytically derived fragment of p170 (data not shown). For comparison, the reactivity of an antiserum raised against rabbit eIF-3 (33) with HeLa and rabbit reticulocyte eIF-3 is shown (Fig. 2C). This serum has strong reactivity with essentially all the rabbit reticulocyte eIF-3 subunits, but reacted less well with the human protein.

The serum to HeLa eIF-3 was used to determine whether a change in eIF-3 could be detected in poliovirus-infected cells. HeLa cells were infected with poliovirus, and a parallel culture was mock infected. Cells were removed at the times indicated in Fig. 3, and extracts were prepared and analyzed on SDS-polyacrylamide gels. When the infected and mock-infected extracts were compared on immunoblots with the antiserum to HeLa eIF-3 (Fig. 3A), no difference was seen in the amount or the mobility of the p170, p115, p47, or p40 subunits at any time point. Furthermore, no additional bands at other mobilities were visualized in the infected-cell extract. This immunoblot also shows that this anti-eIF-3 serum does not react with p220 as does the anti-reticulocyte eIF-3 serum (14, 33). In Fig. 3B, the same extracts were analyzed in immunoblots with an antiserum to p220 (30). In the extracts from infected cells, most of the p220 was gone by 2.5 h postinfection, and the cleavage products appeared. The time course of p220 cleavage in this infection is consistent with that previously observed (14, 30). This blot shows that even though no change in eIF-3 was detected, p220 was cleaved in these infected cells, so that the p220 cleavage activity must have been activated. The anti-

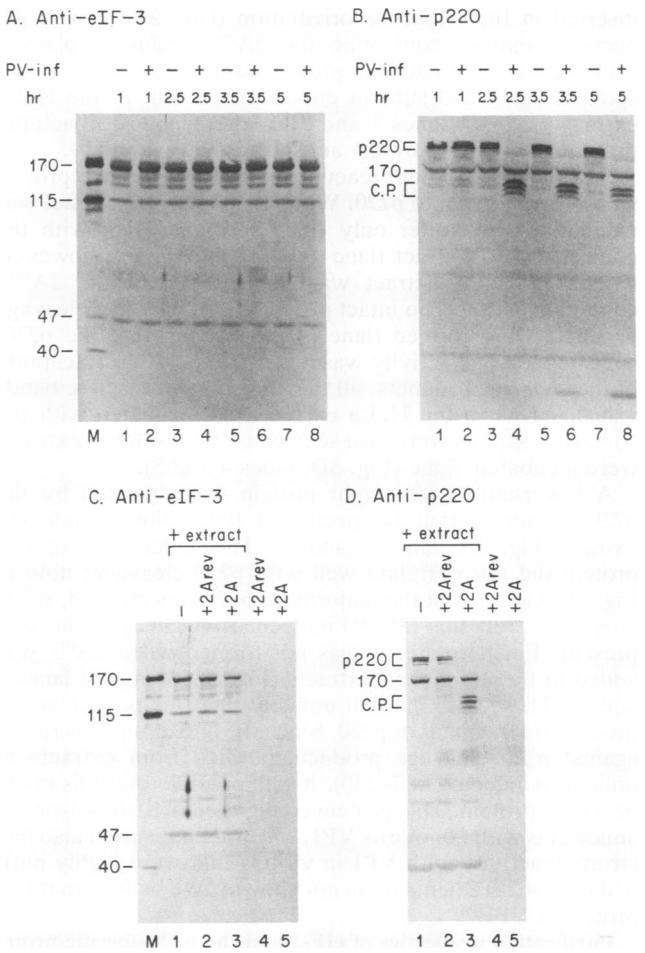

FIG. 3. Immunoblot analysis of eIF-3 in poliovirus-infected and uninfected HeLa cells. (A and B) Poliovirus-infected (lanes 2, 4, 6, and 8) and mock-infected (lanes 1, 3, 5, and 7) HeLa cells were removed at the indicated times, rapidly lysed, and analyzed on SDS-7.5% polyacrylamide gels. (C and D) Postmitochondrial supernatants from HeLa cells were incubated with buffer alone (lane 1) or extracts from *E. coli* containing either pATH-2Arev (lane 2) or pATH-2A (lane 3). pATH-2Arev (lane 5) and pATH-2A (lane 6) extracts were also incubated alone. Reactions were stopped by boiling in SDS gel sample buffer and analyzed on an SDS-7.5% polyacrylamide gel. Proteins were visualized with antisera to human eIF-3 (A and C) or to p220 (30) (B and D). Purified HeLa eIF-3 was included as a marker in blots visualized with the antiserum to eIF-3 (A and C, lane M). Sizes of protein bands ($\times 10^3$) are shown on the left of each gel.

serum was previously shown to cross-react with the 170,000-Da subunit of eIF-3, which is labeled in Fig. 3 (48).

Analysis of eIF-3 after incubation with $2A^{pro}$ in vitro. We also tested whether changes in eIF-3 could be detected after incubation with $2A^{pro}$ in vitro. The source of $2A^{pro}$ was an extract prepared from *E. coli* containing the plasmid pATH-2A. The structure of pATH-2A and characterization of the $2A^{pro}$ activity expressed from this plasmid have previously been described (1, 48). As shown in Fig. 3C, p170, p115, p47, and p40 were visualized in the HeLa extract after probing with the anti-eIF-3 serum. No difference was seen in these subunits when the extract was incubated with buffer alone (Fig. 3C, lane 1), with the control extract made from *E. coli* carrying the plasmid pATH-2Arev in which the 2A gene was

inserted in the antisense orientation (lane 2), or with the bacterial extract containing the $2A^{pro}$-producing plasmid pATH-2A (lane 3). All the protein bands that do not correspond to an eIF-3 subunit either are present in the HeLa extract (Fig. 3C, lanes 1 and 2) or correspond to bacterial proteins recognized by the antiserum (lanes 4 and 5).

Portions of the same reaction mixtures were also probed with the antiserum to p220. When the HeLa cell extract was incubated with buffer only (Fig. 3D, lane 1) or with the control *E. coli* extract (lane 2), p220 was intact; however, when the HeLa extract was incubated with the $2A^{pro}$-containing extract, no intact p220 was seen and the cleavage products were formed (lane 3), confirming that the p220-specific cleavage activity was activated. With the exception of the cleavage products, all the new immunoreactive bands visualized when the HeLa extract was incubated with the bacterial extracts were also seen when the bacterial extracts were incubated alone (Fig. 3D, lanes 4 and 5).

A lower-molecular-weight protein was detected by the p220 antiserum that is specific to the poliovirus-infected extract (Fig. 3B, lanes 6 and 8). The appearance of this protein did not correlate well with p220 cleavage; note in Fig. 3B, lane 4, that the majority of p220 was cleaved, while very little of this 35,000-molecular-weight protein was present. Furthermore, it was not formed when $2A^{pro}$ was added to the uninfected extracts (Fig. 3D, compare lanes 2 and 3). These data made it unlikely that this protein was a proteolytic fragment of p220. Since the antiserum was raised against p220 cleavage products purified from extracts of poliovirus-infected cells (30), it was possible that this could be a viral protein. This protein comigrated in SDS-polyacrylamide gels with poliovirus VP1. The p220 antiserum also had strong reactivity with VP1 in virions that were highly purified on CsCl gradients (data not shown). We believe that this protein is VP1.

Purification properties of eIF-3 and the p220-specific protease. If eIF-3 is the latent p220-specific protease, then the activated protease from infected cells should copurify either with intact eIF-3 or with an eIF-3 subunit. The purification properties of eIF-3 and the p220-specific protease were compared by chromatography on both gel filtration and ion-exchange columns. When an RSW-A cut from uninfected HeLa cells was analyzed by gel filtration on Superose-6, eIF-3 eluted as a protein with a very high molecular weight (Fig. 4). Since it eluted in the nonlinear range of the column, the molecular weight could not be determined accurately, but it is greater than 670,000. The molecular weight of purified eIF-3 has been estimated to be 410,000 by analytical centrifugation (5). The higher apparent molecular weight seen in this RSW-A cut may reflect association of eIF-3 with other proteins in the extract. p220 also eluted as a protein with a high molecular weight, and it also was so large that an accurate molecular weight could not be determined (Fig. 4). This apparent size is greater than the calculated molecular weight for eIF-4F and probably results from interaction of this factor with other proteins in the extract. The size distribution of p220 overlapped with that of eIF-3, but was not identical with it. A similar overlapping, but nonidentical, distribution of eIF-3 and p220 has previously been observed in sucrose gradients run under similar salt conditions (12, 14, 15).

The RSW-A cut from poliovirus-infected cells was also analyzed by gel filtration on Superose-6, and fractions were assayed for the p220-specific cleavage activity. The recovered activity was extremely weak, but a very low level of activity was detected eluting with a molecular weight of about 60,000 (data not shown). The eIF-3 in the RSW-A cut eluted at position similar to that of eIF-3 in the extract from uninfected cells (data not shown). Although eIF-3 eluted in a very broad peak, it was separated from the weak cleavage activity (data not shown). Also analyzed on this column was a postribosomal supernatant (S-200) from poliovirus-infected HeLa cells which in this cell extract contained the most of the p220-specific cleavage activity. As previously reported, the p220-specific protease activity is found in both the S-200 and the RSW-A cut, with some variability in the ratio found in these two subcellular fractions (28). The activity in this subcellular fraction was readily detected eluting over a rather broad range, with the peak eluting with an apparent molecular weight of 55,000 to 60,000 (Fig. 4), much smaller than the molecular weight of eIF-3. These data indicate that the p220-specific cleavage activity is not intact eIF-3, although the active site could possibly reside in an eIF-3 subunit, or a proteolytic fragment of a subunit, which becomes dissociated from the protein complex following activation by $2A^{pro}$ and which is not detectable by antibody. The p220 cleavage products eluted at a molecular weight slightly lower than that of uncleaved p220, but still much larger than the 110,000 to 130,000 molecular weight observed on SDS-polyacrylamide gels. The cleavage products in the RSW-A cut from poliovirus-infected cells eluted at a molecular weight very similar to that observed in the S-200 (data not shown). These data are consistent with the previous observation that the p220 cleavage products sedimented only slightly more slowly than intact p220 on sucrose gradients run in low-salt buffers (12). This suggests that the p220 cleavage products are present in a complex that is not completely disrupted by poliovirus infection.

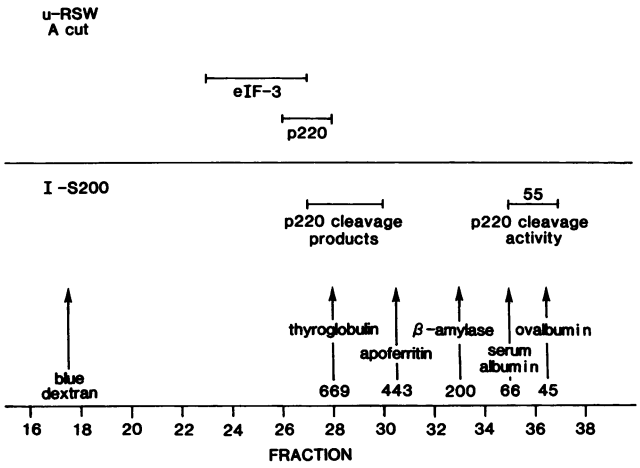

FIG. 4. Gel filtration analysis of extracts from poliovirus-infected and uninfected HeLa cells. An RSW-A cut prepared from uninfected HeLa cells (top panel) and a postribosomal supernatant from poliovirus-infected cells were analyzed on a Superose-6 column. The bars indicate the fractions containing the indicated proteins. When it was possible to estimate the molecular weight of the protein, this is indicated above the bar ($\times 10^3$). The arrows indicate the positions of the molecular weight markers. The molecular sizes of these proteins in kilodaltons are indicated at the bottom of each arrow.

The cellular extracts were also analyzed by anion-exchange chromatography on Mono-Q. Figure 5A shows a p220 cleavage assay of fractions from a Mono-Q column of an RSW-A cut from poliovirus-infected cells. All the p220-specific cleavage activity bound to the column, since no p220

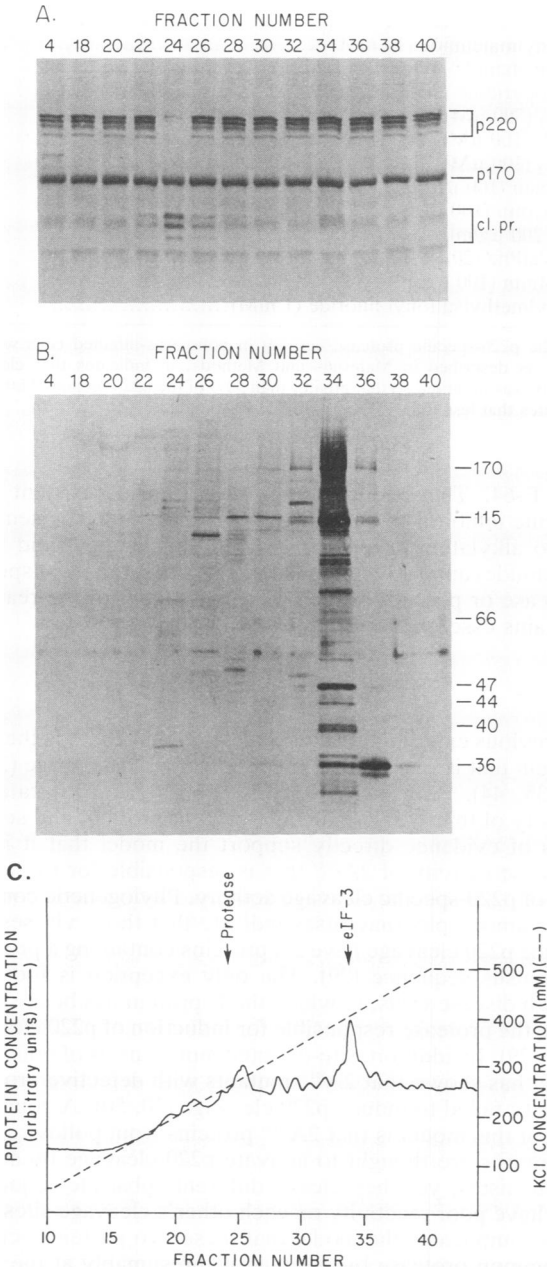

FIG. 5. Separation of eIF-3 and the p220 protease activity on Mono-Q. An RSW-A cut was prepared from poliovirus-infected HeLa cells and analyzed on a Mono-Q column. (A) The indicated column fractions were assayed as described in Materials and Methods. Reactions were analyzed on an SDS-6% polyacrylamide gel, and p220 was visualized in this immunoblot with a p220 antiserum (30). (B) The indicated column fractions were analyzed on an SDS-7.5% polyacrylamide gel, and eIF-3 was visualized in this immunoblot with antiserum raised against rabbit reticulocyte eIF-3 (33). The mobilities of the eIF-3 subunits ($\times 10^3$) are indicated. (C) The A_{280} of the eluate is indicated by the solid line, and the salt gradient profile is the dashed line. The locations of fractions containing p220 cleavage activity and of eIF-3 are marked with arrows.

cleavage is seen in the assay of fraction 4, which contained unbound proteins. Most of the p220 cleavage activity eluted in fraction 24. In the assay of this fraction, most of the p220 substrate was cleaved and the characteristic cleavage products were formed. When the same column was analyzed directly on an immunoblot with an antiserum raised against rabbit reticulocyte eIF-3 (Fig. 5B), the majority of the eIF-3 eluted in fraction 34. No p220 cleavage was observed in the assay of this fraction (Fig. 5A), confirming the gel filtration data that the p220-specific protease separates from intact eIF-3 during purification. On this intentionally overloaded immunoblot (Fig. 5B), a few weakly immunoreactive proteins are visualized with the eIF-3 serum in the active fraction 24. With the possible exception of the 170,000- and 115,000-Da subunits, none of these polypeptides comigrated with eIF-3 subunits. The presence of antigenically detectable p170 or p115 has not been reproducibly observed in column fractions containing the p220-specific cleavage activity. Other weakly immunoreactive proteins in this fraction were also not consistently present in the active fractions from different Mono-Q columns. An additional feature that can be observed on this immunoblot is that, with the possible exception of p66, which reacts only weakly with this antiserum, all eIF-3 subunits are observed in fraction 34 (Fig. 5B). This indicates that none of the subunits are completely separated from the eIF-3 protein complex in poliovirus-infected cells. The analysis of the Mono-Q column is summarized in Fig. 4C. This shows elution of the p220-specific protease at approximately 250 mM KCl, while eIF-3 eluted at about 410 mM KCl. The same column fractions were also blotted with a serum raised to $2A^{pro}$ (1), and as previously reported (31), no $2A^{pro}$ was detected in the fraction containing the p220-specific cleavage activity (data not shown).

Requirement of eIF-3 for p220 cleavage by the activated p220-specific protease. It was previously shown that eIF-3 must be added to a partially purified p220 substrate in order for cleavage of that p220 to be induced by $2A^{pro}$ (48). In those experiments, the position of eIF-3 in the protease cascade depicted in Fig. 1 could not be determined. The activity that catalyzes p220 cleavage in poliovirus-infected cells is thought to represent the activated form of the p220-specific protease (Fig. 1). The clear separation of this protease activity from eIF-3 on Mono-Q (Fig. 5) allowed us to determine whether eIF-3 acts after the activation of this protease. If eIF-3 is the latent precursor of the p220-specific protease, or if its sole function is to promote the formation of this activated protease, then once the protease is activated in the infected cells, eIF-3 should no longer be required for p220 cleavage. Alternatively, if eIF-3 acts after formation of the activated p220-specific protease, then eIF-3 should still be required for p220 cleavage by this protease. To determine which is correct, we prepared an S-200 from poliovirus-infected cells and separated proteins on a Mono-Q column. The fractions were first assayed by incubating them with the crude postmitochondrial supernatant fraction from uninfected HeLa cells to identify the fractions containing the activated p220-specific cleavage activity. When this postmitochondrial supernatant from uninfected HeLa cells, which contained both p220 and eIF-3, was incubated with buffer alone, p220 was seen in the immunoblot (Fig. 6, lane a); however, when it was incubated with the peak active fraction from the Mono-Q column, intact p220 was not observed and cleavage products were formed (Fig. 6, lane b). The Mono-Q fraction contained a few proteins which reacted with the p220 antiserum (Fig. 6, lane f). To determine whether this cleavage is dependent on eIF-3, we then tested

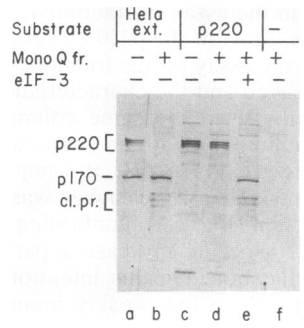

FIG. 6. Both eIF-3 and the p220 cleavage activity are required for p220 cleavage. A postmitochondrial supernatant from uninfected HeLa cells was incubated either with buffer alone (lane a) or with the fraction containing the peak p220 cleavage activity from a Mono-Q column (lane b). Partially purified p220 was incubated with buffer only (lane c), with the peak fraction p220 cleavage activity (lane d), or with the p220 cleavage activity fraction and purified HeLa eIF-3 (lane e). The fraction containing the p220 cleavage activity was also incubated alone (lane f). Reactions were analyzed on an SDS-6% polyacrylamide gel and visualized with a p220 antiserum (30).

the Mono-Q active fraction for activity on a p220 substrate in which p220 had been separated from eIF-3. This substrate was prepared by partial purification of p220 on phosphocellulose and calmodulin Sepharose as described in Materials and Methods. Incubation of this substrate with buffer revealed intact p220 (Fig. 6, lane c). Incubation of the p220 substrate with the activated p220-specific protease-containing Mono-Q fraction (Fig. 6, lane d) again yielded intact p220. This result demonstrated that the partially purified p220-specific cleavage activity from poliovirus-infected cells is not sufficient for cleavage of p220. In contrast, when the substrate was incubated with both the p220-specific cleavage activity and eIF-3, p220 was efficiently cleaved (Fig. 6, lane e), indicating that the p220-specific cleavage activity in poliovirus-infected cell extracts requires eIF-3 for activity. Incubation of the p220 substrate with only eIF-3 did not result in any detectable cleavage of p220 (data not shown). The requirement for eIF-3 observed in this experiment demonstrates that eIF-3 must act after the formation of the activated p220-specific protease formed in poliovirus-infected cell extracts.

We found that p220 binds efficiently to calmodulin Sepharose in the presence of calcium, and this proved to be a useful step in p220 purification. Although the binding of p220 to this column was not investigated in detail, our data suggest that p220 is likely to be a calmodulin-binding protein. This result is not unexpected, since p220 is a substrate for the calcium-dependent proteases calpain I and II (49), and many calpain substrates are also calmodulin-binding proteins (47).

Inhibitors of p220 cleavage. To further characterize the p220-specific protease activity in poliovirus-infected cells, we determined the sensitivity of the p220 cleavage reaction to various protease inhibitors. In these experiments, extracts from poliovirus-infected cells were incubated with uninfected HeLa cell extracts as described in Materials and Methods in the presence of the indicated inhibitor. The results are summarized in Table 1. p220 cleavage was resistant to a number of compounds that characteristically inhibit some serine and/or cysteine proteases, such as aprotinin, phenylmethylsulfonyl fluoride, leupeptin, antipain, and E-64. The cleavage activity was also resistant to a specific inhibitor of calpains, CbzValPhe (32). The sensitivity to alkylating agents (e.g., *N*-ethylmaleimide, and iodoacetamide) and heavy metals indicates that the p220-specific protease or possibly some other component of the reaction contains essential free thiols.

TABLE 1. Effect of inhibitors on p220 cleavage[a]

Inhibitor (concn)	Inhibition[a]
None	–
N-Ethylmaleimide (10 mM)	+
Iodoacetamide (10 mM)	+
Dithiopyridine (500 μM)	+
$ZnCl_2$ (500 μM)	+
$HgCl_2$ (100 μM)	+
$CdCl_2$ (100 μM)	+
Antipain (100 μM)	–
Leupeptin (100 μM)	–
E64 (200 μg/ml)	–
CbzValPhe (20 μM)	–
Aprotinin (100 μg/ml)	–
Phenylmethylsulfonyl fluoride (1 mM)	–

[a] The p220-specific protease activity in poliovirus-infected cells was assayed as described in Materials and Methods. + indicates that cleavage activity was inhibited so that greater than 50% of the p220 remained intact. – indicates that less than 10% of the p220 was intact.

DISCUSSION

Previous experiments have indicated that $2A^{pro}$ is the viral protein that is required for induction of p220 cleavage (3, 24, 29, 35, 44). Protease activity is the only known catalytic activity of this 17,000-molecular-weight protein, and several lines of evidence directly support the model that it is the protease activity of $2A^{pro}$ that is responsible for the induction of p220-specific cleavage activity. Phylogenetic comparisons among picornaviruses indicate that those viruses that induce p220 cleavage have 2A proteins containing a protease consensus sequence (29). The only exception is foot-and-mouth disease virus, in which the L protein has been shown to be the protease responsible for induction of p220 cleavage (2, 8, 29). In addition, site-directed mutagenesis of poliovirus $2A^{pro}$ has shown that $2A^{pro}$ mutants with defective protease activity failed to induce p220 cleavage (20, 50). A complication of this model is that $2A^{pro}$ proteins from poliovirus and rhinovirus are thought to activate p220 cleavage by similar mechanisms, yet they cleave different substrate sequences and have poor reactivity on each other's cleavage sites (40). This complicates the model that these two proteins activate a common protease by cleaving it, presumably at the same site.

We have previously shown that eIF-3 is a cellular protein required for $2A^{pro}$-induced cleavage of p220. Although the role of eIF-3 was not clear from this assay, it presented the possibility that eIF-3 was the cellular protein that is activated in poliovirus-infected cells to become the p220-specific cleavage activity. A prediction of this model is that eIF-3 could be a cleavage substrate for $2A^{pro}$. A simple test of this model is complicated by the fact that eIF-3 is a very large, poorly characterized protein with 7 to 10 polypeptide subunits. With an antiserum to HeLa eIF-3, we were unable to detect an alteration in any of the eIF-3 subunits either in extracts of poliovirus-infected cells or following incubation of a HeLa cell extract with $2A^{pro}$. From these data and data from similar immunoblots, we conclude that no alteration of

eIF-3 could be detected with this antiserum. A limitation of this analysis is that this serum has poor reactivity with p66 and p44. In addition, cleavage of a small proportion of any of these polypeptides or a cleavage that removes only a small number of amino acids would be difficult to visualize by this technique. During chromatography on Mono-Q, eIF-3 in extracts from both infected and uninfected cells eluted at exactly the same salt concentration. This is consistent with no major changes occurring in the structure or subunit composition of eIF-3 during poliovirus infection. The chromatography of eIF-3 on the gel filtration column Superose-6 is somewhat more difficult to interpret, since eIF-3 from both infected and uninfected cells eluted in very broad peaks. The peak appeared to be even broader in extracts from poliovirus-infected cells, and although this could be the result of structural changes in eIF-3, it could also reflect changes in the interactions of eIF-3 with other proteins. In summary, we find no conclusive evidence for any changes in the structure of eIF-3 in poliovirus-infected cells. This is consistent with the results of others in which no change in eIF-3 structure (9, 14) or activity (13) was detected in poliovirus-infected cells.

Separation of intact eIF-3 from the p220-specific cleavage activity was observed on both Superose-6 and Mono-Q columns. This indicates that the activated p220-specific protease is not the eIF-3 complex, and if this activity resides on one of the eIF-3 subunits, it must separate from the intact complex in extracts from infected cells. Immunoblots of the column fractions did not indicate coelution of the p220-specific cleavage activity with an eIF-3 subunit. This analysis was complicated by several factors, including reactivity of the antiserum with non-eIF-3 proteins in the extract and the possibility that a putative activated subunit of eIF-3 was present in low levels in the extract or derived from a subunit which reacts poorly with the antiserum.

The separation of eIF-3 from the p220-specific cleavage activity in infected cell extracts allowed us to determine whether eIF-3 is required for p220 cleavage by the activated p220-specific protease present in poliovirus-infected extracts. The results showed that both eIF-3 and the p220-specific protease are required for p220 cleavage. This experiment indicates that eIF-3 is required in the p220 cleavage reaction at a step that occurs after the formation of the activated p220-specific protease (Fig. 1). It is possible that eIF-3 acts at multiple steps in the cleavage cascade, and the question of whether eIF-3 is also required to form this protease is not addressed by this experiment. This leaves the formal possibility that eIF-3 is the latent cellular protease, but none of the available evidence supports this model, and we believe that the most likely explanation of our data is that eIF-3 and the p220-specific protease activity are separate activities which carry out different roles in the p220 cleavage reaction.

If eIF-3 is not the p220-specific protease, then its role in p220 proteolysis remains to be determined. We previously proposed that the substrate for the cleavage reaction could be an eIF-3–eIF-4F complex (48). An eIF-3–eIF-4F interaction has been previously observed by numerous investigators. eIF-4F subunits have been observed in various preparations of purified eIF-3 (43), and an antiserum raised against purified eIF-3 has been shown to react with p220 (14, 33). Cosedimentation of eIF-3 with p220 (12, 14) and with eIF-4F-associated activities (17, 46) from uninfected HeLa extracts has been observed on sucrose gradients in low-salt buffers. The cosedimentation was disrupted in extracts from poliovirus-infected cells (12, 15, 17). The cap-binding protein and "restoring activity" were shown also to bind to eIF-3 Sepharose (46). These observations are consistent with an eIF-4F–eIF-3 complex serving as the substrate in the cleavage reaction and would be consistent with the requirement for eIF-3 at a step after activation of the protease. There are other possible mechanisms by which eIF-3 could promote p220 cleavage; for example, it could interact with and stabilize or stimulate the activity of the p220-specific protease. Another possibility is that it could bind to and inactivate an inhibitor of the p220 cleavage reaction.

The identity of the p220-specific protease is still not known. Several lines of evidence indicate that this protease is different from $2A^{pro}$ (24, 31), and data in this report indicate that it is unlikely to be eIF-3. We have previously shown that $2A^{pro}$ expressed in a bacterial extract and highly purified eIF-3 will induce the cleavage of a partially purified p220 substrate (48). If the latent form of this protease is present in this reaction as an additional cellular protein, the most likely source of this protein is the partially purified p220 substrate. So far, we have been unable to show separation of such an activity from p220 on a variety of columns (unpublished data), so that if the inactive form of the p220-specific protease is contributed from this source, it must be tightly associated with p220. If such an association exists, however, it must be disrupted in poliovirus-infected cells, since the activated form of the p220-specific protease separates from the p220 cleavage products on both gel filtration (Fig. 4) and phosphocellulose (28) columns.

An additional source of information on the p220-specific protease is its sensitivity to various protease inhibitors. It is of interest that when the inhibitor sensitivities of the p220-specific protease are compared to those of $2A^{pro}$ (23), the patterns are similar. Like $2A^{pro}$ activity, p220 cleavage is inhibited by alkylating agents, suggesting a cysteine protease, but it is not sensitive to a variety of inhibitors that block a spectrum of cysteine or serine proteases. The basis of this similarity is not known, but work is in progress to identify inhibitors that specifically inhibit either $2A^{pro}$ or the p220-specific cleavage activity. The spectrum of protease inhibitor sensitivities for the p220-specific protease, together with a molecular weight of approximately 55,000 to 60,000 does not clearly suggest any known cellular protease.

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