The p220 Component of Eukaryotic Initiation Factor 4F Is a Substrate for Multiple Calcium-Dependent Enzymes†

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ABSTRACT: Eukaryotic initiation factor 4F (eIF-4F) is a multisubunit protein that functions in the first step of the binding of capped mRNAs to the small ribosomal subunit. Its largest polypeptide component, p220, is cleaved following poliovirus infection. This is thought to inactivate eIF-4F function, thereby preventing cap-dependent initiation of translation of cellular mRNAs. In this report, we show that p220 in extracts of uninfected HeLa cells is specifically lost in the presence of calcium. The responsible activities have been partially purified and identified as the calcium-dependent, neutral, cysteine proteases calpains I and II. In addition, a third calcium-dependent activity was resolved from the calpains and also results in the loss of p220. This activity has properties similar to a transglutaminase and copurifies with tissue transglutaminase through several chromatographic steps. None of these calcium-dependent activities appears to mediate p220 cleavage in poliovirus-infected cells.

The processes which regulate the initiation of protein synthesis in eukaryotes are complex and incompletely understood. Eukaryotic initiation factor 4F (eIF-4F) is required for efficient translation of capped, cellular messenger RNAs [for reviews, see Rhoads (1988) and Sonenberg (1988)]. This factor is thought to bind the m^7GTP cap on the 5′ end of mRNAs and to promote binding of the mRNA to the 40S ribosomal subunit (Grifo et al., 1983; Tahara et al., 1981). eIF-4F appears to have the ability to discriminate between different mRNAs (Lawson et al., 1988; Ray et al., 1983). In addition alterations in eIF-4F activity correlate with changes in translational efficiency during oocyte maturation (Huang et al., 1987), heat shock (Duncan et al., 1987; Panniers et al., 1985), mitosis (Bonneau & Sonenberg, 1987), and poliovirus infection (Ehrenfeld, 1984; Etchison et al., 1982; Sonenberg, 1987).

eIF-4F consists of multiple polypeptides. The most well-characterized component is the p25 cap binding protein, also called eIF-4E. This protein can be specifically cross-linked to oxidized 5′-caps on mRNAs in the absence of ATP and magnesium (Sonenberg et al., 1978). It is likely to be the component of the complex which confers the specific recognition of caps. Its gene has been cloned and sequenced from several sources (Altmann et al., 1987; Rychlik et al., 1987a; Sonenberg, 1988), and a site of serine phosphorylation has been determined (Rychlik et al., 1987b). Dephosphorylation of p25 correlates with altered translation rates during mitosis (Bonneau & Sonenberg, 1987) and heat shock (Duncan et al., 1987), but not poliovirus infection (Buckley & Ehrenfeld, 1986). Changes in p25 phosphorylation do not abolish its cap binding activity (Buckley & Ehrenfeld, 1986; Duncan et al., 1987; Hiremath et al., 1989) but may alter its ability to promote translation initiation (Joshi-Barve et al., 1990; Lamphear & Panniers, 1990). Another peptide component of eIF-4F is p220 (Grifo et al., 1983). p220 is visualized on SDS–polyacrylamide gels as a set of several proteins with an average molecular weight of approximately 220 000 (Lloyd et al., 1985). The biochemical contribution of p220 to eIF-4F activity is unclear, but cleavage of this polypeptide during poliovirus infection correlates with the loss of translation of capped, host mRNAs (Etchison et al., 1982; Lloyd et al., 1987). eIF-4F purified by most protocols also contains a polypeptide similar to or identical with eIF-4A. This 45 000-dalton polypeptide cross-links to oxidized caps on reovirus mRNA in the presence of ATP and magnesium (Edery et al., 1983; Grifo et al., 1982, 1983). eIF-4A is an ATP-dependent helicase and may act to remove secondary structure from the 5′ end of the mRNA (Ray et al., 1985).

During poliovirus infection, the expression of a viral protease, called $2A^{pro}$, is required to induce cleavage of p220 (Krausslich et al., 1987; Lloyd et al., 1988; Sun & Baltimore, 1989). During poliovirus infection, this small, cysteine protease cleaves the viral capsid protein precursor from the rest of the viral polyprotein (Toyoda et al., 1986). Although the 2A protease is required for p220 cleavage, it does not catalyze the cleavage reaction itself (Lloyd et al., 1986). Instead, it appears that the viral protease activates a latent cellular protease which catalyzes p220 proteolysis. This latent cellular protease has not been identified. These data prompted a search for cellular proteases which existed in a latent form in the cell, and which exhibited specificity for p220 as a substrate.

In this report, we show that p220 is a substrate for the cytoplasmic proteases calpain I and calpain II, which are normally activated by calcium. In addition, another calcium-dependent enzyme, which appears to be tissue transglutaminase, utilizes p220 as a specific substrate.

Experimental Procedures

Materials. Antipain, leupeptin, pepstatin, aprotinin, and E-64 [*N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]agmatine] were purchased from Boehringer Mannheim Biochemicals, and *N*-ethylmaleimide, putrescine, ethylamine,

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and methylamine were purchased from Sigma. CbzValPheH (Mehdi et al., 1988) was the gift of Dr. Martin Rechsteiner. Goat anti-guinea pig liver tissue transglutaminase was the gift of Dr. Peter Davies (Murtaugh et al., 1983).

Assay for Calcium-Dependent p220 Degradation. Fractions to be assayed were incubated in the presence of 8 mM $CaCl_2$ with p220 substrate which had been partially purified to separate it from endogenous degrading activities. This purification involved preparation of a ribosomal salt wash fraction (Brown & Ehrenfeld, 1979), from which p220 was concentrated by precipitation with 40% saturated ammonium sulfate followed by dialysis against 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 5% glycerol. In control reactions, this p220 substrate was completely stable in the presence of calcium. Incubation was performed in 20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.5 mM dithiothreitol, and 8 mM $CaCl_2$ for 90 min at 30 °C. In some assays, p220 was not separated from endogenous activity, and calcium was added directly to the postmitochondrial supernatant. The conditions used in these assays were 4 μL of HeLa postmitochondrial supernatant, 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 8 mM $CaCl_2$ (or as indicated), and incubated at 30 °C for 90 min (or the indicated time).

Partial Purification of Calcium-Dependent Activities. Cell pellets containing approximately 5×10^9 cells were resuspended in H buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 7 mM 2-mercaptoethanol) plus 10 mM KCL. The cells were lysed in a Dounce homogenizer, and a high-speed supernatant was prepared as previously described (Brown & Ehrenfeld, 1979). This supernatant was loaded on a 30-mL P-11 phosphocellulose column (Whatman) which had been equilibrated in buffer H. Fractions containing the unadsorbed protein were pooled; the KCl concentration was adjusted to 75 mM and loaded on a 25-mL phenyl-Sepharose column (Pharmacia) which had been equilibrated with buffer H containing 75 mM KCl. Bound protein was eluted from the column in 5 mM HEPES, pH 7.4, and 0.5 mM EDTA. Activity on p220 was observed in both the unbound protein fractions and the eluate. Protein in each of these pools was concentrated by precipitation with 80% ammonium sulfate, and dialyzed against H buffer containing 75 mM KCl. The concentrated protein from the eluate was loaded on a Sephacryl S-200 column (Pharmacia), and the activity peak eluted from the column with proteins of molecular weight between 100 000 and 150 000. Active fractions were pooled, diluted 1:1 with H buffer, and loaded on a Mono-Q 5/5 prepacked column (Pharmacia) which had been equilibrated in H buffer. Protein was then eluted in a 0–0.8 M KCl gradient in H buffer with the activity eluting in two sharp peaks at 230 and 400 mM KCl. The concentrated protein which did not bind to the phenyl-Sepharose was loaded on a Sephacryl S-200 column. It eluted with proteins of molecular weight between 50 000 and 100 000. A separate aliquot of the protein that did not bind the phenyl-Sepharose column was loaded on a Mono-Q column, and bound proteins were eluted with a 0.075–1 M KCl gradient. The activity eluted at a KCl concentration of about 0.45 M.

Calpain, Calpastatin, and Antibodies against Calpain. The calcium-dependent proteases (calpains) I and II and their inhibitory protein, calpastatin, were purified from bovine heart as described previously (Croall & DeMartino, 1984; DeMartino et al., 1988). Polyclonal antisera were raised in rabbits against purified bovine calpain II (Beckerle et al., 1987). The antiserum used reacts with the 80 000-Da subunit of calpain II and 26 000-Da subunit of each protease and cross-reacts with the 84 000-Da subunit of calpain I. The antiserum cross-reacts with all mammalian calpains that have been examined thus far including rat, monkey, and human.

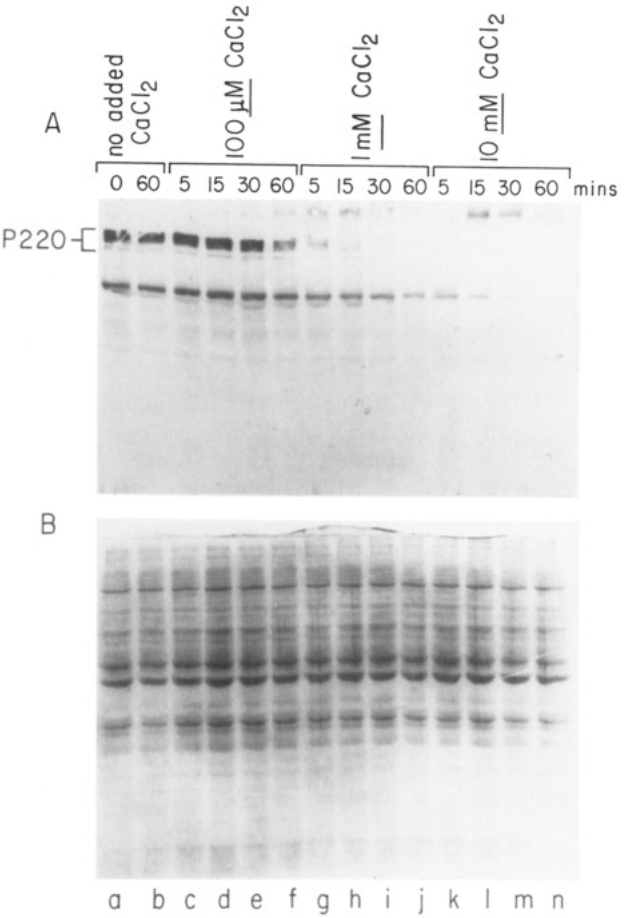

FIGURE 1: Loss of p220 from HeLa cell extracts following addition of calcium. $CaCl_2$ was added to HeLa cell extracts at the indicated final concentrations and incubated at 30 °C for various times. A portion of each reaction was run on a 6% SDS–polyacrylamide gel, and p220 was detected by immunoblotting (panel A). Portions of the same reactions were run on a 10% SDS–polyacrylamide gel, and total protein was visualized by staining with Coomassie brilliant blue (panel B).

RESULTS

Calcium-Dependent Loss of p220. During the course of studies designed to characterize the p220 component of eIF-4F, it was observed that addition of calcium to crude extracts from HeLa cells caused a rapid disappearance of p220. A time course of this effect at several calcium concentrations is shown in Figure 1A. p220 was identified by probing blots of SDS–polyacrylamide gels of total cytoplasmic proteins with a polyclonal antiserum prepared against p220 polypeptide isolated from poliovirus-infected HeLa cells (Lloyd et al., 1987). In the absence of added calcium, p220 is visualized in immunoblots as four closely spaced bands with an average molecular weight of about 220 000. An additional protein of M_r 150 000 is also detected with this antiserum, but it is not known whether it represents a related, cross-reacting protein or simply a contaminant that was present in the immunogen used to raise the antiserum. After 1 h in 100 μM $CaCl_2$, there is only slight loss of p220. However, at higher concentrations of $CaCl_2$, the loss of p220 was extremely rapid. Note the appearance of very high molecular weight immunoreactive material which is visible in the earlier time points. Portions of the reactions shown in Figure 1A were analyzed on a sep-

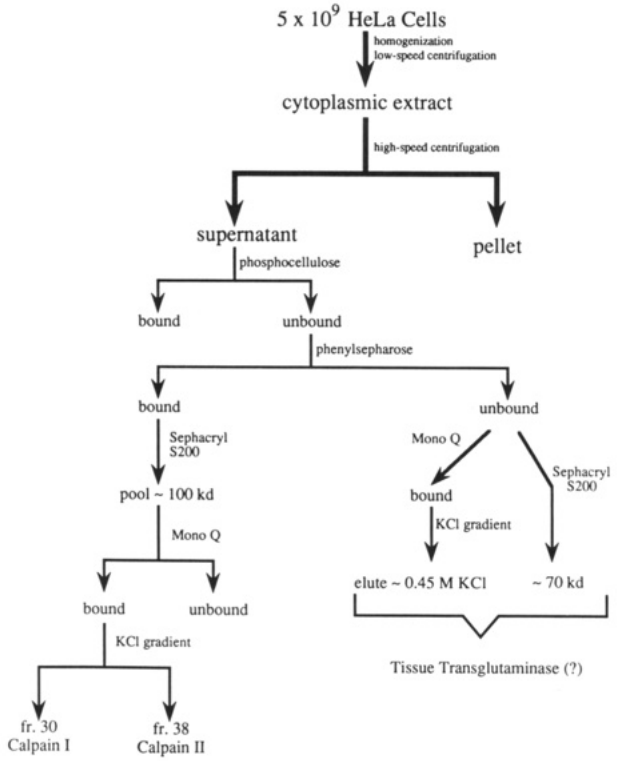

FIGURE 2: Summary of partial purification of p220-specific calcium-dependent activities. Activities were partially purified from HeLa cell extracts as described under Experimental Procedures.

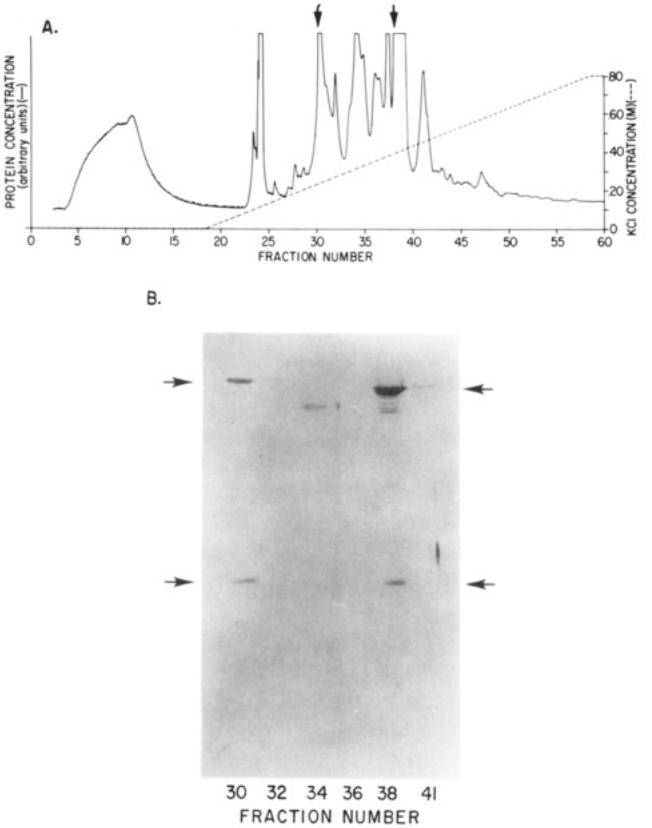

FIGURE 3: Copurification of p220-degrading activity with calpains I and II. Pooled material containing p220-degrading activity was loaded on a Mono-Q 5/5 prepacked column, and bound protein was eluted in a 0–0.8 M KCl salt gradient. In panel A, the solid line is the profile of protein eluting from the column as monitored by the UV absorbance at 280 nm. The dashed line is the salt concentration. The arrows indicate the p220-degrading activity in fractions 30 and 38. Panel B is an immunoblot in which fractions from this column were run on a 10% SDS–polyacrylamide gel and probed with antibody raised to bovine calpain II which cross-reacts with all mammalian calpains tested. The arrows indicate the large and small calpain subunits.

arate gel which was stained with Coomassie brilliant blue, and shown in Figure 1B. Very little change in the overall protein pattern was observed under conditions when p220 was completely absent. Separate immunoblots were performed to determine whether rapid loss in the presence of calcium is a general property of protein synthesis initiation factors. These immunoblots indicated that $CaCl_2$ had no effect on the stability of another eIF-4F component, eIF-4A, or any of the three polypeptides of eIF-2 (not shown). Thus, it appears that the p220 subunit of eIF-4F is a specific substrate for a calcium-dependent activity or activities present in the HeLa cell cytoplasm. This effect is not specific to HeLa cells, since similar results were obtained with BHK cell extracts and a commercially obtained rabbit reticulocyte lysate (data not shown). In addition, no effect on p220 stability was observed following incubation with other divalent cation salts, including $MgCl_2$, $MnCl_2$, $ZnCl_2$, or $CdCl_2$, suggesting that this effect is specific for calcium (data not shown).

Characterization of Activities That Act on p220. In order to characterize the activity which caused the loss of p220, an assay was developed by which calcium-dependent p220 degradation activities could be purified. This assay required a p220 substrate which was free of endogenous calcium-dependent activity. Such a substrate was obtained by subcellular fractionation, since the ribosomal salt wash fraction, which has been previously reported to contain the majority of p220 (Etchison et al., 1982), showed no loss of p220 when incubated with calcium. During subcellular fractionation, the calcium-dependent, p220-specific activity was found only in the post-ribosomal supernatant, and this provided the starting material for development of a purification protocol. The purification procedure is described in detail under Experimental Procedures and summarized in Figure 2. All the p220-specific activity flowed through the phosphocellulose column with the unbound protein. However, during chromatography on phenyl-Sepharose, activity was found both with unbound protein and also with the bound protein that eluted in a buffer step containing no salt. The pooled fractions from both the phenyl-Sepharose-unbound material and the eluate were tested for the presence of the calcium-dependent protease calpain by immunoblotting with an antibody raised to calpain II (Beckerle et al., 1987). Calpain was detected only in the protein that had bound the column, but not in the unbound protein. This suggested that the two pools contained different activities, and they were further analyzed separately.

The activity which bound the phenyl-Sepharose column, and contained calpain, was chromatographed on a Sephacryl S-200 gel filtration column. The activity eluted with protein with a molecular weight of 100 000–150 000, and these active fractions contained calpain as visualized in immunoblots (data not shown). These active fractions were pooled and chromatographed on Mono-Q. Two peaks of activity eluted from this column which is summarized in Figure 3. Figure 3A shows the profile of total protein eluting from the Mono-Q column as assayed by the UV absorbance at 280 nm. The two arrows indicate two peaks of p220-degrading activity at fractions 30 and 38. This activity coelutes from the column with the calcium-dependent protease, calpain, as visualized by immunoblotting (Figure 3B). It has previously been observed that anion-exchange chromatography will separate the two forms of this protease, calpains I and II (Croall & DeMartino, 1983; Yoshimura et al., 1983) (discussed below).

Table I: Effect of Protease Inhibitors on $CaCl_2$-Dependent p220 Degradation by Mono-Q Column Fractions[a]

inhibitor (concentration)	fraction 30	fraction 38
none	–	–
antipain (100 μM)	+	+
leupeptin (100 μM)	+	+
N-ethylmaleimide (10 mM)	+	+
pepstatin (1.5 mM)	–	–
aprotinin (100 μg/mL)	–	–
E-64 (200 μg/mL)	+	+
CbzValPheH (20 μM)	+	+
ethylamine (100 mM)	ND	–

[a] Cleavage of the p220 substrate was determined as described under Experimental Procedures. "+" indicates cleavage activity was inhibited so that greater than 50% of the p220 substrate was intact as estimated by immunoblotting. "–" indicates less than 10% p220 was intact. "ND" indicates experiment not done.

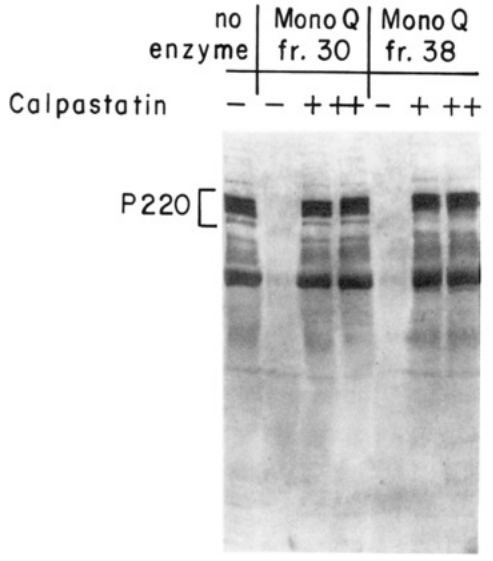

FIGURE 4: Inhibition of p220 degradation by calpastatin. Portions of the active Mono-Q fractions were assayed for p220-degrading activity in the presence and absence of purified calpastatin, a calpain-specific inhibitor. The presence of calpastatin in the protease reactions is indicated above the gel lanes. Reactions run in the lanes marked + and ++ contained 1 and 3 μL of purified calpastatin, respectively. The position of p220 in this immunoblot probed with an anti-p220 serum is indicated on the left.

From silver-stained gels of these fractions, it was estimated that the partially purified calpains I and II constitute 5–10% of the total protein in these samples.

The activities observed in the Mono-Q fractions were further characterized by testing their sensitivity to a variety of protease inhibitors (Table I). This sensitivity spectrum is consistent with the protease sensitivity data reported for calpain (Yoshida et al., 1983). In addition, calpain activity is regulated in vivo by a highly specific protein inhibitor, calpastatin (DeMartino & Croall, 1984; Murachi, 1983). This protein has not been observed to inhibit any other protease (Murachi, 1983). The p220-degrading activity in the Mono-Q fractions was strongly inhibited by purified calpastatin (Figure 4). The copurification through several ion-exchange, hydrophobic, and gel filtration columns, plus the demonstrated inhibition by the highly specific calpain inhibitor, calpastatin, provides strong evidence that the activities observed are calpains. Finally, independent preparations of highly purified calpain I and calpain II (Croall & DeMartino, 1984; DeMartino et al., 1988) were fully active for p220 degradation. Figure 5 shows that both calpain I and calpain II induced degradation of p220 at calcium concentrations previously determined for their activities. The activity was completely dependent on calcium

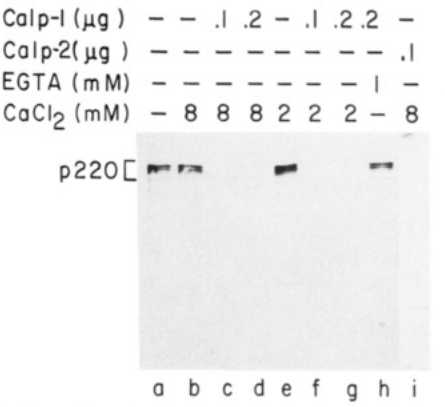

FIGURE 5: Calcium-dependent degradation of p220 by purified calpains I and II. Purified calpains I and II (Croall & DeMartino, 1984; DeMartino et al., 1988) were assayed as described under Experimental Procedures. Reactions contained the indicated amount of calpain I (Calp-1) or calpain II (Calp-2) and the indicated concentration of $CaCl_2$ or EGTA.

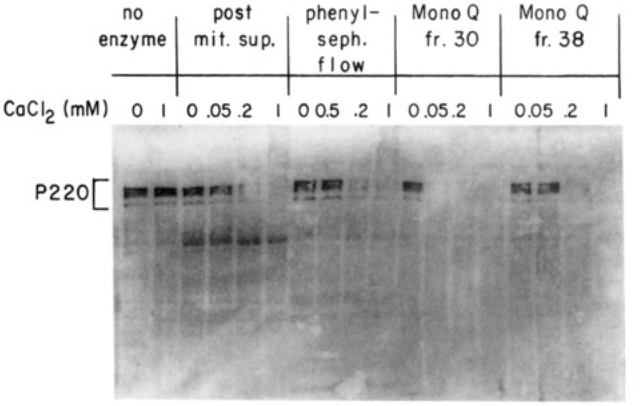

FIGURE 6: Calcium dependence of p220-specific reactions. Fractions containing activities that use p220 as a substrate were dialyzed against 20 mM HEPES, pH 7.4, 50 mM KCl, and 7 mM 2-mercaptoethanol to remove chelators. The indicated fractions were assayed as described under Experimental Procedures, except that reactions contained the indicated final concentrations of calcium. The position of p220 in this immunoblot is indicated.

(Figure 5, lane h, and data not shown). The utilization of p220 as a substrate by both enzymes is consistent with the previously reported overlap in substrate specificities (Wang et al., 1989).

The calcium concentration required for activation of the enzymes in the Mono-Q fractions was determined (Figure 6). The activity in fraction 30 required a much lower concentration of calcium than the activity in fraction 38. On the basis of this calcium dependence, the activity in fraction 30 is most likely to be calpain I while the activity in fraction 38 is likely calpain II. This has been confirmed by the observation that the large calpain subunit in fraction 30, but not fraction 38, reacted with a calpain I epitope-specific antibody in immunoblots (data not shown). This is consistent with the large calpain subunit of calpain I (fraction 30) being slightly larger than the large subunit of calpain II (fraction 38) visualized in the immunoblot (Figure 3B) (Aoki et al., 1986; Imajoh et al., 1988). In addition, it is well established that calpain I elutes from anion-exchange columns at a lower salt concentration than calpain II (Croall & DeMartino, 1983; Yoshimura et al., 1983).

As described above, not all of the calcium-dependent, p220-specific activity bound to phenyl-Sepharose. No calpain was detected in immunoblots of the unbound pooled fractions (data not shown), suggesting that this activity is distinct from the calpains. The purification properties of this activity were

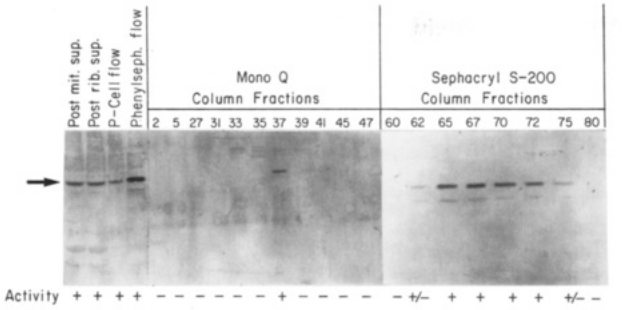

FIGURE 7: Copurification of p220-specific activity with tissue transglutaminase. A p220-specific activity which did not bind phenyl-Sepharose was partially purified as described under Experimental Procedures and summarized in Figure 2. Pooled material from various stages in this purification was run on 7.5% SDS–polyacrylamide gels and probed in immunoblots with antibody raised to guinea pig liver tissue transglutaminase. The position of tissue transglutaminase is indicated by the arrow on the left, and the presence of activity causing the loss of p220 is indicated below each lane. "+" indicates that less than 25% of the p220 was intact when the column fraction was assayed as described under Experimental Procedures. "+/–" indicates that greater than 75% of the p220 was visualized intact in the immunoblot but that high molecular weight material was formed. The central portion of the figure shows column fractions obtained from a Mono-Q column from which bound protein was eluted in a 75–800 mM KCl gradient in H buffer. The right portion of the figure shows those fractions containing protein eluting from a Sephacryl S-200 column near the p220-specific activity. For both columns, the starting material was pooled, concentrated fractions from the phenyl-Sepharose flow.

further characterized as described under Experimental Procedures and summarized in Figure 2. A portion of the unbound material was concentrated and chromatographed on a Sephacryl S-200 column. This activity eluted later than calpain-associated activity, very close to the position of bovine serum albumin observed in a separate standardization run. This represents a lower molecular weight than that observed for the calpains when they were analyzed on the same column. A separate portion of the unbound protein was chromatographed on a Mono-Q column. The activity bound to the column and eluted in the salt gradient at about 450 mM KCl. That is somewhat higher salt than was required to elute calpain II in a separate run using the same column and buffers. In addition, the activity which did not bind phenyl-Sepharose was not inhibited by leupeptin or antipain which are known inhibitors of the calpains. In reactions performed simultaneously, these compounds did inhibit partially purified calpains I and II (not shown).

The calcium dependence of the activity that did not bind phenyl-Sepharose is very similar to that of both the crude extract and calpain II (Figure 6). Note in Figures 1 and 6 that high molecular weight material is generated in assays of the activity that flowed through both phenyl-Sepharose and the postmitochondrial supernatant, but not in assays of the calpains. This high molecular weight immunoreactive material was consistently seen in reactions with crude extract and the phenyl-Sepharose-unbound fractions, but the amount was variable and was usually quantitatively less than the amount of p220 observed in the control lanes. We do not know if this reduced and variable amount of high molecular weight material occurs because some of the material is subject to proteolysis or if it is the result of the immunoblotting procedure, such as reduced reactivity with our antiserum or failure of this extremely high molecular weight material either to enter the resolving gel efficiently during electrophoresis or to transfer completely to the nitrocellulose during blotting. The calcium-dependent generation of high molecular weight material suggested that this activity could be a transglutaminase.

Table II: Effect of Inhibitors on p220 Degradation by the Postribosomal Supernatant[a]

inhibitor (concentration)	postribosomal supernatant
none	–
N-ethylmaleimide (10 mM)	+
$ZnCl_2$ (50 μM)	+
putrescine (5 mM)	+
methylamine (10 mM)	+
ethylamine (50 mM)	+
antipain (100 μM)	–
leupeptin (100 μM)	–
E-64 (200 μg/mL)	–
pepstatin (1.5 mM)	–
aprotinin (100 μg/mL)	–

[a] $CaCl_2$ was added directly to the postmitochondrial supernatant, and p220 cleavage was assayed as described under Experimental Procedures. "+" and "–" are used as described in Table I.

Several properties of the activity in the phenyl-Sepharose flow are consisent with it being tissue transglutaminase, which is the major intracellular transglutaminase (Folk, 1980). Protein that reacted with an antibody prepared against guinea pig tissue transglutaminase was present in the material that did not bind phenyl-Sepharose, and the immunoreactive protein coeluted with the p220-specific activity from both Sephacryl S-200 and Mono-Q chromatography columns (Figure 7). The molecular weight of this activity as estimated from the Sephacryl S-200 column was approximately 70000, which is near the 76000 monomer observed for guinea pig tissue transglutaminase (Folk & Cole, 1966; Ikura et al., 1988). This activity requires a high concentration of calcium for activity, as has been described for tissue transglutaminase (Lorand & Conrad, 1984).

In order to determine which of the activities described above is responsible for loss of p220 observed in the crude extract, the inhibitor sensitivity of the p220-specific activity in the postmitochondrial supernatant was tested. Transglutaminases are known to contain essential cysteines, and their activity is sensitive to thiol blocking reagents. They are also inhibited by small amines which are thought to compete with the cross-linking reaction (Folk, 1980; Folk & Cole, 1966; Lorand & Conrad, 1984). The sensitivity of the calcium-dependent activity present in the postribosomal supernatant to various inhibitors of transglutaminases and proteases is shown in Table II. This activity was sensitive to thiol blocking reagents and small amines, but not to protease inhibitors, as is characteristic of a transglutaminase. This characteristic spectrum of inhibitor sensitivities together with the fact that the activity in crude extract resembles the activity separated from calpains on phenyl-Sepharose both in its calcium concentration dependence and in the generation of high molecular weight products suggests that the transglutaminase-like activity is likely to be the major contributor to the activity observed in the crude extract. The observation that the calpains do not appear to mediate degradation of p220 in the crude postmitochondrial supernatant is not surprising, since it has been seen previously that calpain activity is sometimes not observed in crude extracts and that the calpain activity is revealed after partial purification to separate it from the endogenous inhibitor, calpastatin (Murachi, 1983; Waxman & Krebs, 1978).

Discussion

We have shown that p220 is a substrate for at least three calcium-dependent enzymes. Two of these enzymes are calpains I and II. Calpains (EC 3.4.22.17), also known as calcium-activated neutral protease, CANP, are nonlysosomal, calcium-dependent, cysteine proteases having a neutral pH

optimum [for review, see Mellgren (1987), Murachi (1983), and Suzuki (1987)]. Two forms of calpain, known as calpains I (μCANP) and II (mCANP), have been observed in a variety of mammalian tissues. Both are heterodimers. The large subunits are encoded by separate but related genes, which encode proteins with predicted molecular weights of 84 000 and 80000 for calpains I and II, respectively (Aoki et al., 1986; Imajoh et al., 1988). The large subunits have four domains, including one with sequence similarity to papain and other cysteine proteases and another containing four E-F hand consensus sequences for calcium binding sites (Ohno et al., 1984). The 30000-dalton small subunit of both calpains is the product of the same, single-copy gene (Emori et al., 1986; Miyake et al., 1986). Although both calpains absolutely require calcium for activity, different concentrations are required by the two forms. Calpain I requires micromolar concentrations of calcium, while calpain II requires millimolar calcium concentration for activity. Although calpains have been shown to cleave a variety of substrates in vitro and are ubiquitously distributed in mammalian tissues, their physiological functions remain to be elucidated (Mellgren, 1987; Murachi, 1983). At this time, too little is known about the functions of these proteases to speculate about the possible significance of their ability to catalyze p220 cleavage.

We have found an additional p220-specific activity which is distinct from the calpains, and which is likely to be the principal activity observed in the crude extract. On the basis of the observed physical properties of the enzyme, the high molecular weight products of the reaction, and inhibitor sensitivities, this activity appears to be tissue transglutaminase. Transglutaminases (EC 2.3.2.13) catalyze the formation of covalent ϵ-(γ-glutamyl)lysine cross-links in proteins [see Folk (1980) and Lorand and Conrad (1984) for reviews]. The protein–protein cross-linking activity of transglutaminases results in the generation of high molecular weight protein products. In the presence of low molecular weight polyamines, transglutaminases also catalyze the exchange of a primary amine for ammonia at the γ-carboxamide group of protein-bound glutamine residues, resulting in conjugation of polyamine to protein. Transglutaminases are widely distributed in tissues and body fluids, and a number of immunologically distinct forms have been described. One of these, tissue transglutaminase, is a 76 000-dalton enzyme present in the soluble fraction of cells (Folk & Cole, 1966; Ikura et al., 1988). Although activity is absolutely dependent on millimolar concentrations of calcium, no E-F hand consensus sequences are apparent in its primary structure. Like the calpains, tissue transglutaminase is widely distributed in mammalian tissues, but its physiological function is unknown.

The specificity of these activities toward p220 was discovered while studying the degradation of p220 that occurs during poliovirus infection. However, the polio-induced p220 cleaving activity has different inhibitor sensitivities and purification properties fom both calpains I and II and transglutaminase (unpublished data). In addition, none of the calcium-dependent activities give the characteristic p220 cleavage products observed in poliovirus-infected cells (Figure 6), suggesting that the polio-induced cellular protease is likely to be a distinct enzyme from the calcium-dependent enzymes described here.

The fact that cleavage of p220 correlates with inhibition of translation in poliovirus-infected cells suggests that alteration of p220 can regulate translation. Our observation that p220 is a substrate for calpains and tissue transglutaminase, together with other reports that p220 is a substrate for protein kinase C (McMullin et al., 1988; Tuazon et al., 1989), suggests the possibility that p220 could be a calcium-responsive component of the translational apparatus. Although the relationship to the effects we observe on p220 is not clear, there have been several recent reports indicating that calcium is involved in translation. Recent experiments suggest that calcium is required for efficient translation in vivo (Brostrom et al., 1989a; Chin et al., 1987). Interestingly, this requirement is lost following thermal or chemical stress (Brostrom et al., 1989b). Others have reported that calcium is required for stability of the 43S preinitiation complex (Kumar et al., 1989).

As a technical note, cell lysates are routinely treated with calcium and micrococcal nuclease prior to their use for in vitro translations (Pelham & Jackson, 1976). In a commercially obtained rabbit reticulocyte lysate, p220 was lost following incubation with calcium under the conditions normally used to assay loss of p220 (data not shown). In addition, immunoblots indicate that tissue transglutaminase was present in the lysate (unpublished data). The procedure of Pelham and Jackson (1976) uses a very short incubation at low temperature (10 min at 18 °C). When this protocol was followed precisely, no alteration in the p220 band pattern was detected in immunoblots (unpublished data). In the absence of more extensive, quantitative data, it may be advisable to limit the calcium pretreatment as much as possible.

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