Induction of DNA synthesis in isolated nuclei by cytoplasmic factors: Inhibition by protease inhibitors

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ABSTRACT Cytoplasmic extracts from spontaneously proliferating and mitogen-activated lymphoid cells contain a protein factor called ADR (activator of DNA replication) that induces DNA synthesis in isolated quiescent nuclei. ADR-containing preparations have proteolytic activity, as indicated by their ability to degrade fibrin in a plasminogen-independent and plasminogen-dependent manner. In addition, aprotinin, a nonspecific protease inhibitor, abrogates ADR-induced DNA synthesis in a dose-dependent fashion. Preincubation studies demonstrated that the effect of aprotinin is not due to its suppressive effects on the nuclei themselves. Other protease inhibitors such as leupeptin, p-aminobenzamidine, and N-α-tosyllysine chloromethyl ketone are also inhibitory, but soybean trypsin inhibitor is without effect. ADR activity can be removed from active extracts by adsorption with aprotinin-conjugated agarose beads and can be recovered by elution with an acetate buffer (pH 5). These findings are consistent with the interpretation that the initiation of DNA synthesis in resting nuclei may be protease dependent and, further, that the cytoplasmic stimulatory factor we have called ADR may be a protease itself.

It has been known for many years that nuclear DNA synthesis is under cytoplasmic control (1). Using cell-free systems, a number of investigators have shown that cytoplasmic extracts from proliferating cells contain a protein(s) that can initiate nuclear DNA synthesis (2–9). We have reported such cytoplasmic intermediate(s) in spontaneously proliferating and mitogen-activated lymphoid cells (7, 8). This factor, which we have called activator of DNA replication (ADR), is a heat-labile protein of Mr >100,000. It is not detectable in resting cells. Although it activates isolated quiescent nuclei, it has no effect on intact cells. ADR is not species specific. Furthermore, ADR appears to mediate an intracellular mitogenic signal in the interleukin 2 (IL-2) T-cell activation pathway (9). Das (6) has described a cytoplasmic intermediate in epidermal growth factor-stimulated 3T3 fibroblasts that shares many functional and physicochemical properties with ADR. In addition, Benbow and Ford (2) reported a similar cytoplasmic stimulatory factor in early embryonic tissue. The description of ADR-like proteins in a number of cell types and experimental systems suggests a significant role for these factors in cellular proliferation and differentiation.

To our knowledge, no information is available as to the biochemical nature of ADR or the ADR-like factors in other cell types (2–6). We now report that ADR preparations have proteolytic activity and that the functional activity of ADR can be significantly inhibited by aprotinin and a number of other protease inhibitors. The inhibition of ADR-induced DNA synthesis was not due to any direct effects of aprotinin on the isolated resting nuclei. Rather, the protease inhibitor appears to interact with ADR itself.

MATERIALS AND METHODS

Cell Cultures and Preparation of Cytoplasmic Extracts. MOLT-4, a spontaneously proliferating human T-cell leukemia line, was maintained as described (7). Peripheral blood lymphocytes were obtained from young, healthy donors, isolated, cultured, and stimulated with phytohemagglutinin at 10 μg/ml (Sigma) as described (7). Cytoplasmic extracts were prepared by hypotonic lysis and Dounce homogenization (7). Homogenates were centrifuged at 3000 × g for 10 min. Supernatant was collected and adjusted to 0.1 M sucrose/10 mM KCl before ultracentrifugation at 140,000 × g for 60 min (type 65 rotor, Beckman). Partial purification of ADR was by ammonium sulfate fractionation and Amicon ultrafiltration (7).

Preparation of Isolated Nuclei. Adult frogs (Xenopus laevis) were purchased from Nasco ( Ft. Atkinson, WI). Isolated nuclei were prepared by the detergent lysis method of Benz and Strominger (10) as described (7). Since ADR lacks species specificity (7), frog nuclei were used for the routine assay.

Assay for DNA Synthesis in Isolated Nuclei. Assays were performed in triplicate in 96-well microtiter plates (Falcon) as described (7). Nuclei (2 × 10⁶ nuclei) were cultured with reaction buffer [0.25 M sucrose/25 mM Hepes, pH 7.8/25% (wt/vol) dextran (Sigma)] and cytoplasmic extract. The total volume of all wells was 100 μl. Fifty microliters of incorporation mixture containing 0.5 mM dATP, 0.5 mM dGTP, and 0.5 mM dCTP; 5 mM ATP; 12.5 mM phosphoenolpyruvate; 10 units of pyruvate kinase per ml; 38 mM Hepes (pH 7.8); 10 mM KCl; 125 mM MgCl₂; and 2 mM dithiothreitol were added to each well. Each well then received 3 μl of [methyl-3H]dTTP (1 mCi/ml, 40–70 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). The plates were incubated at 37°C for 90 min. Time-zero measurements were also performed to measure nonspecific background radioactivity; these values were subtracted from the respective postincubation counts. These conditions have been shown to result in maximal [3H]dTTP incorporation. At the end of the incubation period, the reactions were stopped, and nuclei were harvested as described (7).

Abbreviations: ADR, activator of DNA replication; IL-2, interleukin 2; SBTI, soybean trypsin inhibitor; TLCK, N-α-tosyllysine chloromethyl ketone.

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Source of IL-2. Purified, lectin-free IL-2 was obtained from Electro-Nucleonics (Oak Ridge, TN). A 1:6 dilution of the IL-2 preparation contained 640 half-maximal units per ml. The IL-2 preparation was validated by its ability to maintain IL-2-dependent T-cell lines.

Stimulation of Lymphoblasts with IL-2. Peripheral blood lymphocytes were collected and cultured with phytohemagglutinin as described above. After 5 days, the lymphoblasts were collected and recultured at 1–1.5 × 10^6 cells per ml with either complete medium alone or complete medium plus 2% (wt/vol) IL-2 for another 48 hr. The cells were then collected, and cytoplasmic extracts were prepared from both cultures as described above.

Preincubation Experiments. Frog spleen nuclei were prepared as described above and were incubated with aprotinin at 250 µg/ml for 15 min at 37°C. Control nuclei were incubated with nuclei buffer in a similar fashion. The nuclei were then centrifuged at 2000 × g for 10 min, recounted, and resuspended at 2 × 10^9 nuclei per ml with nuclei buffer. ADR was added, and DNA synthesis was measured in the usual fashion.

Assay of Serine Protease Activity. Proteolytic activity was based on the hydrolysis of 125I-labeled fibrin, coated onto tissue culture wells. Fibrinogen, free of plasminogen, was purified according to published methods (11) and radiolabeled by lactoperoxidase-catalyzed iodination as described (12, 13). Human plasminogen was purified as described by lysine-Sepharose 4B affinity chromatography (13). The 125I-labeled fibrinogen was placed in tissue culture wells (10 µg/cm², 500–700 cpm/µg) and dried at 45°C for 72 hr. Following drying, the 125I-labeled fibrinogen-coated wells were incubated at 37°C for 2.5 hr in medium supplemented with fetal bovine serum. Thrombin and other factors contained in the serum converted 125I-labeled fibrinogen to insoluble 125I-labeled fibrin. Each well was washed twice with PBS (0.01 M, pH 7.4) and washed an additional time just prior to the assay with 0.1 M Tris-HCl (pH 8.1). The assay incubation mixture contained 0.02% Triton X-100, 1 ml of 0.1 M Tris-HCl (pH 8.1), an aliquot of ADR, and where indicated, 5–10 µg of purified plasminogen. The radioactivity released from the wells was determined by direct γ counting.

Adsorption of ADR with Agarose Beads. Slurries containing plain agarose beads, soybean trypsin inhibitor (SBTI)-conjugated agarose beads, or aprotinin-conjugated agarose beads (all from Sigma) were centrifuged at 170 × g for 10 min and washed three times with the hypotonic extract buffer. Ovalbumin (10 mg/ml) (Sigma) was mixed with the beads at a ratio of 2 ml of ovalbumin/1 ml of packed beads. This was done to prevent nonspecific sticking of protein to the agarose itself. The beads were incubated at 37°C for 30 min on a rocker platform (Lab Line, Melrose Park, IL). The beads were rinsed on filter paper five times with extract buffer and resuspended in extract buffer. The beads were centrifuged at 170 × g for 10 min. Supernatant was removed, and MOLT-4 cytoplasmic extract was added at a ratio of 2 ml of extract/1 ml of packed beads. The beads were then incubated at 37°C for 30 min on a rocker platform. The beads were centrifuged at 170 × g for 10 min. The supernatant was removed and assayed for ADR activity as described above. The beads were stored overnight at 4°C with a small amount of extract buffer to prevent drying.

Elution of ADR from Agarose Beads. A modification of the method of Baugh and Travis (14) was used. Syringes (5 ml) were packed with scrubbed nylon wool (Fenwal Laboratories, Deerfield, IL) up to the 1-ml mark and washed with extract buffer. Aprotinin-agarose and plain agarose beads prepared from adsorption assays were added to separate columns and allowed to settle. Each column was washed extensively with extract buffer before the addition of elution buffer (0.05 M sodium acetate, pH 5.0/0.4 M NaCl). Eight 1-ml fractions were collected from each column and dialyzed. Protein content was estimated spectrophotometrically using a Coomassie blue dye-binding procedure (Dura Research Products, Cheshire, CT).

Calculation of Specific Activity. Units of ADR activity = ½[(cpm incorporated by ADR-stimulated nuclei – background cpm)/(cpm incorporated by nuclei alone – background cpm)]. Therefore, 1 unit of ADR activity represents a 2-fold increase in [3H]dITTP incorporated into nuclei incubated with ADR over that incorporated by nuclei alone.

RESULTS

Protease Activity in ADR-Containing Extracts. To demonstrate protease activity, we utilized radiolabeled fibrin as substrate. The results are shown in Fig. 1. By 21 hr of incubation, we observed significant degradation of fibrin even in the absence of a source of plasminogen. By 48 hr, 2900 counts (above background) were obtained (data not shown). When plasminogen was added, there was marked augmentation of proteolysis in a time-dependent manner. Plasminogen alone was proteolytically inactive. In this experiment, urokinase was included as a highly active plasminogen-dependent positive control, and by 21 hr, we achieved similar levels of activity with ADR. These results demonstrate that the ADR-containing preparations contain a neutral serine proteolytic activity that can both degrade fibrin directly and convert plasminogen to plasmin.

Inhibition of ADR Activity by Aprotinin. Nuclei were isolated from adult frog splenocytes and incubated with cytoplasmic extracts prepared from proliferating MOLT-4 cells, in the presence and absence of increasing doses of aprotinin and SBTI. As shown in Fig. 2A, aprotinin significantly inhibited ADR activity in the MOLT-4 extracts in a dose-dependent manner. In contrast, no significant inhibition of ADR-induced DNA synthesis occurred with SBTI over a

![Fig. 1. Proteolytic activity of ADR preparations.](image-url)
derived from incubated Increasing from IL-2-stimulated extract Points representative are comparable also extracts As cells, (non-neoplastic) activity derived from the ADR source of inhibitors SBTI showed ketone FIG. 2. Effects of Other Protease Inhibitors on ADR Activity. Isolated frog splenocyte nuclei (2 × 10⁶ nuclei) were incubated with 20 μl of MOLT-4 extract and increasing concentrations of aprotinin (●), TLCK (●), leupeptin (●), and p-aminobenzamidine (●). Results are from a representative experiment. Each point is the mean of three samples; error bars are the SD.

dine were tested for the ability to inhibit ADR activity. The concentration of the protease inhibitors ranged from 31.5 to 250 μg/ml. Fig. 3 demonstrates the inhibition profile of these various agents. The doses of aprotinin, TLCK, and leupeptin required to achieve 50% inhibition of ADR activity in this experiment were very similar (=25 μg/ml) whereas p-aminobenzamidine exhibited significantly less inhibitory effect (ED₅₀ = 200 μg/ml).

Failure to Inhibit ADR-Induced DNA Synthesis by Preincubation of Resting Nuclei with Aprotinin. To determine whether aprotinin may be directly affecting the nuclei themselves, quiescent frog nuclei were prepared in the usual manner and preincubated with aprotinin at 250 μg/ml. This dose was used because it had been shown to cause complete inhibition of DNA synthesis when added to nuclei and MOLT-4 extracts in mixed cultures. After 15 min, the nuclei were washed, cultured with ADR, and assayed for DNA synthesis in the usual fashion. As shown in Table 1, the nuclei were unimpaired in their ability to respond to ADR after pretreatment with aprotinin.

Adsorption of ADR Activity on Aprotinin-Conjugated Agarose Beads. We next tested the ability of insolubilized

**Table 1. Lack of inhibition of ADR activity by aprotinin preincubated with resting frog nuclei**

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<td></td>
<td>808 ± 383</td>
<td>22,931 ± 923</td>
<td>0 ± 30</td>
<td>29,225 ± 1,253</td>
<td>818 ± 224</td>
<td>27,301 ± 1,268</td>
<td>1,475 ± 373</td>
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Nuclei were isolated from frog splenocytes and preincubated with aprotinin (250 μg/ml) or buffer. Control nuclei were kept on ice during the preincubation period. Pretreated and control nuclei were then cultured with 20 μl of MOLT-4 extract, and cultures were assayed for ADR activity in the usual fashion. Results are from a representative experiment and are expressed as the mean of three samples ± SD.

**Fig. 2. Effect of aprotinin and SBTI on ADR activity. (A)** Increasing doses of aprotinin (solid line) and SBTI (dotted line) were incubated with 2 × 10⁶ frog splenocyte nuclei and 20 μl of ADR derived from proliferating MOLT-4 cells. Results are from a representative experiment. (B) Same as A, except 50 μl of cytoplasmic extract from IL-2-stimulated lymphoblasts was used as the source of ADR. Points are the mean ADR activity of three samples; error bars are the SD.

**Effects of Other Protease Inhibitors on ADR Activity.** Four protease inhibitors including aprotinin, N-α-tosyllysine chloromethyl ketone (TLCK), leupeptin, and p-aminobenzamidine could also inhibit cytoplasmic signals derived from normal (non-neoplastic) cells, we repeated these experiments using extracts from IL-2-activated human lymphoblasts as the source of ADR. As shown in Fig. 2B, similar results were obtained. As before, aprotinin significantly inhibited ADR activity derived from the IL-2-stimulated lymphoblasts. SBTI showed only a minimal suppressive effect, which was not statistically significant. In all subsequent experiments cytoplasmic extracts from MOLT-4 cells were used because of the ease of obtaining large amounts of ADR from this source.

**Fig. 3. Effect of protease inhibitors on ADR activity.** Isolated frog splenocyte nuclei (2 × 10⁶ nuclei) were incubated with 20 μl of MOLT-4 extract and increasing concentrations of aprotinin (●), TLCK (●), leupeptin (●), and p-aminobenzamidine (●). Results are from a representative experiment. Each point is the mean of three samples; error bars are the SD.
aprotinin to adsorb ADR activity from active MOLT-4 extracts. MOLT-4 extract was incubated with aprotinin-conjugated, SBTI-conjugated, or plain (control) agarose beads. At the end of the adsorption procedure, the mixtures were centrifuged, and the supernatants were tested for residual ADR activity. Results from a representative experiment are presented in Fig. 4. Adsorption with plain or SBTI-conjugated agarose beads had no effect on ADR activity contained in MOLT-4 extracts. However, adsorption with aprotinin-conjugated beads resulted in a significant loss of ADR activity. In this experiment, for example, 75% of the ADR activity was removed by adsorption with aprotinin-agarose.

Elution and Recovery of ADR Activity from Aprotinin-Conjugated Agarose Beads. Plain and aprotinin-conjugated agarose beads were incubated with MOLT-4 extract as described above. After centrifugation, supernatants were collected and set aside. Columns were prepared from the packed beads and washed. The columns were then eluted with 0.4 M NaCl/0.05 M sodium acetate, pH 5. Eight 1-ml fractions were collected, dialyzed against extract buffer (pH 7.8), and tested for ADR activity in the usual assay. Fig. 5 shows the results of a representative experiment. In this experiment, adsorption with aprotinin-conjugated agarose beads removed approximately 70% of the ADR activity in MOLT-4 extract. As shown in Fig. 5, we were able to recover the ADR activity in fractions 2–4 eluted from these columns. In preliminary experiments, we have studied the eluate fractions in an NaDdSO4/PAGE system in which plasminogen and casein have been incorporated as substrate. Proteolytic activity is associated with a band at 100 kDa in fractions with ADR activity. As expected, none of the fractions eluted from the control agarose columns contained significant ADR activity.

The starting protein concentration in the ADR preparation was 4.88 mg/ml. The protein content of the eluate fractions 1–4 of Fig. 5 was 0.475 μg/ml, 0.475 μg/ml, 0.325 μg/ml, and 0.75 μg/ml, respectively. There was no detectable protein in fractions 5–8 or in any of the eight fractions eluted from the plain agarose columns. We were able to achieve a 4-fold, 11-fold, 13-fold, and 50-fold increase in specific ADR activity, respectively, in fractions 1–4 eluted from the aprotinin-agarose column, as compared to the starting preparation.

![Fig. 4. Adsorption of ADR activity from MOLT-4 extracts by aprotinin-conjugated agarose beads. Cytoplasmic extract was prepared from MOLT-4 cells and cultured with 2 × 10⁵ frog splenocyte nuclei before (—) and after adsorption with aprotinin-conjugated (A—A), SBTI-conjugated (O—O), or plain (•—•) agarose beads. Results are from a representative experiment. Points represent the mean of three samples; error bars are the SD.](image)

![Fig. 5. Elution of ADR activity from aprotinin-conjugated agarose beads. ADR was adsorbed onto aprotinin-conjugated (○—○) or plain (control) agarose (■—■) beads. The beads were eluted with acetate buffer (pH 5), and fractions were collected. Samples (50 μl) of each eluate fraction were cultured with 2 × 10⁵ frog splenocyte nuclei and assayed for ADR activity. Results are from a representative experiment. Points are the mean of three samples; error bars represent SD.](image)

**DISCUSSION**

Proteolytic enzymes may be important in the regulation of cell proliferation (15). Several studies have demonstrated direct mitogenic effects of proteases on intact cells (16–18). Moreover, protease inhibitors can suppress cellular proliferation (19–23). All these studies involve effects on whole cells, with proteolytic activation of cell surface membranes.

In the present study, we have obtained evidence supporting a role for cellular proteases in the intracellular regulation of cell growth. We first showed that the cytoplasmic factor ADR that induces DNA synthesis in isolated quiescent nuclei is associated with proteolytic activity. However, because ADR has not yet been purified to homogeneity, it was necessary to perform additional experiments making use of a battery of protease inhibitors. Using a cell-free assay, we have demonstrated that the induction of DNA synthesis in isolated quiescent nuclei by ADR can be abrogated by the protease inhibitors aprotinin, TLCK, p-aminobenzamidine, and leupeptin, but not SBTI. The inability of aprotinin to inhibit ADR-induced DNA synthesis when preincubated with resting nuclei suggests that this agent has no direct suppressive or toxic effects on the nuclei themselves. The fact that we could adsorb ADR activity from active MOLT-4 extracts with aprotinin-conjugated agarose beads demonstrates that the ADR–nucleus interaction is protease dependent. Most important, the ability to elute ADR activity from these columns means that aprotinin is interacting with ADR itself.

Aprotinin is a serine esterase inhibitor that reversibly inhibits a variety of proteolytic enzymes, including trypsin, chymotrypsin, plasmin, kallikrein, and intracellular proteases (24). As indicated above, the ability of ADR to bind to aprotinin-agarose beads and the elution of ADR from such beads strongly suggests that ADR is a protease. The direct demonstration that ADR preparations exhibit proteolytic activity supports this contention. The ability of TLCK to interfere with ADR activity is also consistent with this interpretation, but also raises another possibility that TLCK can also react with protein kinases (25, 26), and our preparations of ADR also exhibited tyrosine kinase activity. However, neither aprotinin nor the other protease inhibitors used in this study have been reported to have anti-tyrosine
kinase activity, and we have been unable to detect such activity as well. In any event, it remains a theoretical possibility that the panel of protease inhibitors might also inactivate any nonproteolytic enzymes containing partially homologous active sites (for example, serine residues). An extensive review of the literature by computer failed to identify such activity for the panel of protease inhibitors used in this study, with the exception of the TLCK data referenced above. However, the direct determination of enzymatic activity of ADR purified to homogeneity when available, should unequivocally resolve this issue.

A role for intracellular proteases in lymphocyte stimulation has been suggested by several investigators. Ku et al. (22) reported the presence of an intracellular, arginine-specific serine enzyme that is synthesized in higher quantities following mitogenic stimulation of B cells. Furthermore, Nishizawa et al. (27) and Kishimoto et al. (28) reported that during the activation of B lymphocytes by anti-immunoglobulin, membrane-bound serine protease splits a cytoplasmic precursor protein into an active 45-kDa factor. This active cytoplasmic factor induces the phosphorylation of nonhistone nuclear proteins (29, 30). Our results, however, differ from their findings in that the active cytoplasmic stimulatory factor in our system appears to be the protease itself. Furthermore, neither of these studies demonstrated that the appearance of protease activity was functionally related to DNA synthesis itself; thus, it is not clear whether these proteins would be capable of inducing DNA replication in a cell-free system.

The mechanism by which protease inhibitors block nuclear DNA synthesis is presently unclear. It has been suggested that protease inhibitors suppress cell growth by inhibiting protein synthesis directly (31). However, Grayzel et al. (20) demonstrated that TLCK significantly inhibits lymphocyte transformation without a major effect on protein synthesis. The results from our studies, which utilize preformed nuclear activation factors, would also rule against this possibility.

Another role for proteases in the activation of cells for proliferation has been postulated by Brown et al. (32, 33). These investigators reported that nuclei of Chinese hamster cells can be activated for DNA synthesis by treatment with trypsin and suggested that this involves the direct activation of a DNA polymerase. It is possible that ADR could exert its effect via activation of a DNA polymerase, or indeed, of other enzyme systems involved in activation for DNA replication, or DNA replication itself, such as tyrosine kinases or topoisomerases.

It has also been speculated (16) that proteolytic enzymes can mimic the stimulatory effect of growth factors by cleaving cell surface receptors and inducing conformation changes similar to those resulting from the interaction between the receptor and the growth factor itself. One could postulate similar conformational alterations at the level of the nuclear membrane, as an explanation of the ADR effect.

Regardless of underlying mechanism, the results presented here, in conjunction with our previous findings (7–9), suggest that proteases or enzymes with similar active sites are important in the transduction sequence by which exogenous mitogens (phytohemagglutinin, Con A) or growth factor (IL-2) induce lymphocyte proliferation. From the nature of the detecting assay for ADR, which involves its effect on DNA synthesis in isolated quiescent nuclei, it is clear that it is at the terminal end of the chain of events leading from cell surface alterations to nuclear activation. Future studies require not only the elucidation of its precise mechanism of action, but also an attempt to understand how these late events are related to early signals such as cell surface receptor autophosphorylation, ionic fluxes, and alterations in cyclic nucleotide levels.

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