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Initiation of Lymphocyte DNA Synthesis

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Abstract The initiation of DNA replication in T lymphocytes appears to be regulated by two distinct activities: one associated with proliferation which mediates initiation, and another associated with quiescence which blocks initiation. Activated lymphocytes and proliferating lymphoid cell lines produce an activity, termed ADR, which can initiate DNA replication in isolated, quiescent nuclei. ADR is heat-labile, has protease activity or interacts closely with a protease, and is distinct from the DNA polymerases. ADR activity is absent in quiescent lymphocytes and appears in mitogen-stimulated lymphocytes after IL-2 binding. The generation of active ADR appears to be mediated by phosphorylation of a precurser which is present in resting cells. Nuclei from mitogen-unresponsive lymphocytes fail to initiate DNA replication in response to ADR, of potential importance in the age-related decline of immunity.

Quiescent lymphocytes lack ADR and synthesize an ADR-inhibitory activity. The ADR inhibitor is a heat-stable protein which suppresses the initiation of DNA synthesis, but is ineffective at suppressing elongation once DNA strand replication has begun. Nuclei from several neoplastic cell lines fail to respond to the ADR inhibitor, which may play a role in the continuous proliferation of these cells. At least one of these neoplastic cell lines produces both ADR and an inhibitory factor. These findings suggest that the regulation of proliferation is dependent on the balance between activating and inhibitory pathways.

Key words: lymphoid cell lines, ADR, polymerase, mitogen, IL-2

THE INITIATION OF DNA SYNTHESIS IS THE ENDPOINT OF MULTIPLE MITOGENIC PATHWAYS

A number of different mechanisms acting in concert are required to initiate cell proliferation. For some cells a single external signal, such as the binding of a growth factor to its cognate receptor, appears sufficient to initiate the intracellular processes necessary to mediate the transition from quiescence to active proliferation. In other cells, the process requires multiple external signals. In T lymphocytes, the initial proliferative signal is provided by the binding of the T cell antigen receptor to a complex of antigen peptide and MHC protein on the surface of an antigen-presenting cell. This signal, which mediates the exit from quiescence, initiates the synthesis of a number of proteins by the T cell, including IL-2 and its cognate receptor. The binding of IL-2 serves as an obligatory second signal for T cell proliferation and mediates the passage through late G_1 and the initiation of DNA synthesis.

A number of intracellular signal transduction mechanisms are activated by cell surface receptors. One relatively well-studied system is the interactions of receptors with G proteins, which couple receptors with effector molecules (reviewed in [1]). G proteins couple receptors with a number of different effector mechanisms, including the hydrolysis of phosphatidylinositol bisphosphate, the activation of adenylate cyclase, and the activation of cGMP phosphodiesterase. Another prominent signal transduction mechanism associated with mitogenesis is the activation of receptor-associated tyrosine kinase activity (reviewed in [2]). While numerous combinations of external signals and signal transduction mechanisms are involved in initiating cell proliferation, all initial stimuli must eventually mediate one common response, the initiation of DNA replication.

The decision to replicate cellular DNA is for most cells an irreversible commitment to divide. This decision, which is made in the G_1 period of the cell cycle, has been termed the start point [3,4]. Once past this point, cells complete DNA replication and proceed through mitosis without

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pause. Nondividing cells lie between mitosis and the start point in either G_1 or G_0, depending upon the cell's metabolic state. An exception are oocytes, which are blocked in prophase of the first meiotic division. Thus for nearly all cells the key replicative decisions are made at the start point, and defects in these decisions are implicated in proliferative disorders including neoplasia and senescence.

While the eukaryotic DNA polymerases and several accessory proteins involved in replication have recently been characterized, much remains to be discovered about eukaryotic DNA replication. The complex of proteins which contains the polymerases and mediates DNA strand replication is termed the replisome [5]. Among the replisome constituent proteins are DNA polymerase delta [6–8], which replicates the leading strand DNA; PCNA (proliferating cell nuclear antigen), which is a processivity factor for DNA polymerase delta [9–11]; DNA polymerase alpha, which replicates the lagging DNA strand [12–14]; DNA primase, which synthesizes the RNA primer required for DNA polymerase alpha [15,16]; and an associated helicase activity which unwinds the DNA double helix at the replication fork [17]. The eukaryotic replisome undoubtedly contains many more components, considering that the prokaryotic replisome contains over 20 identified proteins. Several groups have identified additional eukaryotic protein factors which are required for the in vitro replication of plasmids containing the SV40 origin by a mixture of cellular and virus-derived proteins [18,19]. However, the relationship between SV40 plasmid replication and eukaryotic DNA replication is not straightforward, since the replication of these plasmids is dependent upon the multifunctional SV40 large T antigen and the viral replication origins. Thus, very little is known about proteins which regulate eukaryotic DNA synthesis, and even less is known about the mechanisms responsible for the initiation of this process.

A CYTOSOLIC ACTIVITY IN PROLIFERATING CELLS REGULATES THE INITIATION OF DNA SYNTHESIS

An important early question in the study of cell proliferation was whether the control of replicative activity was a property of the nucleus itself or was mediated by cytoplasmic factors. When nuclei from quiescent cells were injected into actively proliferating cells [20] or when a quiescent cell was fused with an actively proliferating cell [21], the quiescent cell nucleus became active and initiated DNA replication. This supported the idea that proliferating cells synthesized soluble, intracellular factors which could act upon the nucleus and stimulate DNA synthesis. Cytoplasmic extracts from spontaneously proliferating cell lines [22], early frog embryos [23], and EGF-stimulated fibroblasts [24] were later shown to initiate DNA synthesis in isolated nuclei. These soluble factors appeared to be common to a number of proliferating cells.

We have used peripheral blood lymphocytes (PBL) and transformed lymphocyte cell lines as model systems to study the activity and regulation of molecules which directly regulate DNA replication. The initial experiments tested the ability of cytoplasmic extracts from actively dividing lymphocytes and spontaneously proliferating lymphoid cell lines to induce DNA synthesis in isolated quiescent nuclei [25]. Extracts from human T cell lymphoblastoid cells (MOLT-4), murine plasmacytoma cells (P3X63Ag8.653), human B lymphoma cells (RPMI 8392), and mitogen-stimulated normal human lymphocytes all induced DNA synthesis in the nuclei as measured by the incorporation of ^{3}H-deoxythymidine triphosphate (^{3}H-dTTP). Nuclei incubated without extract and extracts incubated without nuclei did not incorporate ^{3}H-dTTP. Incorporation is not species specific, as extracts from human cells which stimulated nuclei from quiescent human PBL to incorporate ^{3}H-dTTP stimulated equivalent incorporation into nuclei from murine and amphibian cells. This allowed the routine use of easily obtainable and durable nuclei from frog spleen cells. Other laboratories have used frog spleen nuclei in similar assays and likewise found no species specificity, indicating that this activity has been conserved through vertebrate evolution [21,23,26]. Extracts from nonproliferating cells, such as nonstimulated normal human lymphocytes, had no activity; thus, this activity is unique to dividing cells. These results show that proliferating cells contain a cytoplasmic factor that can induce in vitro DNA synthesis in isolated nuclei and thus may be involved in the induction of in vivo DNA synthesis. This factor or these factors have been provisionally termed "Activator of DNA Replication" (ADR).

An initial concern was that the assay measured DNA repair in addition to or instead of DNA replication. A number of observations show

that this is not the case. Under identical experimental conditions, replicative "forks" and "eyes" in the DNA of stimulated nuclei were seen by electron microscopy [22,23]. Incorporation requires the presence of all four deoxyribonucleotides and energy in the form of ATP. Pulse-chase experiments with ^{3}H-dTTP resulted in the label appearing transiently in small DNA fragments (3–5 S) and then shifting to higher MW DNA. Finally, ADR-mediated ^{3}H-dTTP incorporation is completely blocked by antibodies to PCNA [27], a protein which specifically associates with DNA polymerase delta and greatly increases the processivity of the enzyme in replicating the leading DNA strand [10,11]. Current evidence indicates that most eukaryotic DNA repair is performed by DNA polymerase beta, with perhaps some contribution by DNA polymerase alpha, but that DNA polymerase delta is active only in DNA replication [6,13,14,28].

A second concern was that ADR activity was simply one or more of the DNA polymerase enzymes, which are found primarily in the cytosolic fraction when cells are homogenized by mechanical disruption or solubilized by detergent. This was not easily resolved, as ADR activity requires DNA polymerase activity; thus inhibitors of the latter also inhibited the former. However, both aprotinin and cell-derived ADR-inhibitory factors, both of which inhibit ADR activity by $>90\%$, have no effect on DNA polymerase activity (Coffman et al., submitted; Katz and Cohen unpublished, Coffman unpublished). More directly, ADR activity and DNA polymerase activity elute as partially overlapping but distinctly separate peaks from a Mono Q anion exchange column, an aprotinin-agarose column, and a preparative isoelectric focusing apparatus (F. Coffman, unpublished data). Thus, while ADR activity is dependent upon DNA polymerase activity and ADR may interact with the polymerase enzymes, it appears to be a distinct protein or protein complex.

ADR activity could also be detected in cytoplasmic extracts from IL-2-responsive lymphocyte populations that had been stimulated with IL-2 [29]. The ADR response paralleled the proliferative response in both dose and kinetic studies, suggesting that ADR is a direct mediator of IL-2-stimulated T cell DNA replication. The addition of dexamethasone, an inhibitor of IL-2 production, to PHA-stimulated lymphocytes resulted in an inhibition of proliferation and a concomitant decrease in ADR activity in these cells. Both decreases were abolished when exogenous IL-2 was added to the cultures. These results suggest that in T lymphocytes the appearance of ADR activity is dependent upon IL-2, and is not a delayed consequence of the original PHA stimulation.

ADR IS COMPLETELY INTRACELLULAR

To determine if ADR was secreted, supernatant from RPMI 8392 cells was collected and dialyzed. The dialysate was then either diluted or concentrated up to tenfold in each direction and incubated with nuclei. No ADR activity could be detected. This was not due to loss of ADR through dialysis, as ADR is completely retained by the dialysis membranes with MW cutoffs of 12,000–14,000 daltons.

ADR-containing extracts were also tested for activity on intact cells. Intact frog spleen cells were incubated with extracts from RPMI 8392 cells. This extract, even when concentrated, failed to induce DNA synthesis. Addition of Con A to these cells stimulated DNA synthesis; thus the inability of the intact cells to respond to ADR was not due to a proliferation defect. This failure to induce DNA synthesis was not due to species specificity, as cytoplasmic extracts from proliferating murine cells failed to induce DNA synthesis in intact murine lymphocytes. Thus, ADR is not secreted and has no effect on intact cells, but functions entirely as an intracellular mediator [25].

CHARACTERIZATION OF ADR

ADR-containing extracts which were treated with trypsin failed to induce DNA synthesis in isolated nuclei, suggesting that the factor responsible for induction of DNA synthesis is a protein or complex of proteins [22,25]. ADR activity was stable at 4°C for 24 h and remained intact after freeze-thawing and lyophilization/reconstitution [25], but was inactivated by incubation at 60°C for 20 min [30]. Amicon ultrafiltration studies indicated that ADR has a molecular weight greater than 100,000 daltons. Ammonium sulfate fractionation of the ultrafiltration retentate precipitated the ADR activity between 30 and 50% ammonium sulfate saturation. All of the ADR activity in the 30–50% ammonium sulfate fraction binds to Mono Q anion exchange resin, and can be eluted by a 0–0.5 M NaCl gradient as two distinct peaks of activity (Coffman, unpublished). These two peaks may represent two forms of ADR, and experiments are in

progress to better define the components of these peaks.

ADR-ASSOCIATED PROTEASE ACTIVITY

Several lines of evidence indicate that protease activities play important roles in DNA replication. Proteases have been found to stimulate proliferation both in intact cells [31,32] and in isolated nuclei [33]. A number of protease inhibitors have been shown to block DNA replication in intact cells [34,35] and in in vitro systems [36]. Cellular protease activities have been described which correlate with DNA synthesis [37,38]. Finally, several important regulatory molecules are proteolytically destroyed during the course of cell cycle regulation. The p53 tumor antigen is degraded just prior to the initiation of DNA synthesis [39], and cyclins A and B, which modulate the activity of the cdc2 34kD kinase, are destroyed just prior to mitosis [40].

ADR-containing extracts were assayed for protease activity by using fibrin as a substrate, both alone and in the presence of plasminogen, a second substrate which could amplify the initial proteolysis [41]. Significant fibrin degradation was observed following 21 h of incubation, and the addition of plasminogen markedly enhanced this event. Plasminogen alone had no effect. A positive correlation was seen between the amount of ADR activity and protease activity in a number of extracts.

A series of protease inhibitors were assayed for their ability to block ADR activity. Aprotinin was most effective, and inhibited $>95\%$ of the ADR activity at 250 μg/ml. Leupeptin and TLCK were also effective inhibitors of ADR activity. Para-aminobenzamidine was less effective, as 250 μg/ml blocked 50% of the ADR activity. Soybean trypsin inhibitor (SBTI) at comparable concentrations had no effect on ADR activity, indicating that the inhibitor specificity of the ADR-related protease is somewhat different from trypsin. The ability of protease inhibitors to block ADR activity was due to an effect on the soluble components and not a direct effect on the nuclei, as nuclei that were preincubated with inhibitory concentrations of aprotinin and then washed were still able to initiate DNA synthesis in response to ADR.

These results suggested that a protease activity is required for ADR activity, and that the protease activity is either physically associated with ADR (as one molecule or in a complex), or is a separate molecule. To test these possibilities, MOLT-4 cytoplasmic extracts were incubated with aprotinin-agarose, SBTI-agarose, or unconjugated agarose beads. The majority of ADR activity was bound by aprotinin-agarose, and could be eluted by a pH 5 acetate buffer. Unconjugated or SBTI-conjugated agarose had no effect on ADR activity; nor could activity be eluted from these beads. The binding and elution of ADR activity from the aprotinin columns is evidence that either ADR itself is a protease or is tightly associated with a protease. We have recently bound and eluted DNA polymerase activity from this column at 4°C by a very shallow NaCl gradient, and the peak of DNA polymerase activity is distinct from but overlaps the peak of ADR activity (F. Coffman, unpublished). Since aprotinin does not inhibit DNA polymerase activity, this may mean that ADR (as a protease or as a complex with a protease) binds to the DNA polymerase enzymes.

ACTIVATION OF AN ADR PRECURSER

Freshly isolated, nonstimulated lymphocytes are quiescent and contain no detectable ADR activity. Following mitogen stimulation, the lymphocytes begin to proliferate and ADR activity appears in cytosolic extracts made from these cells. Both proliferation, as measured by thymidine incorporation, and ADR activity reach maximal levels 3 days after Con A stimulation [25]. The appearance of ADR activity following mitogen stimulation could result from de novo protein synthesis or from the covalent modification of a pre-existing protein. The latter possibility was tested by incubating extracts from resting lymphocytes with a membrane preparation from proliferating cells, removing the membranes by centrifugation, and assaying the extracts for ADR activity. Incubation with membranes from proliferating cells stimulated the appearance of ADR activity, while little or no activity was seen with extracts incubated with membranes from quiescent cells or with membranes incubated with buffer alone to control for protein which could be solubilized out of the membrane fraction into the soluble phase [42]. This induction of ADR activity is inhibited by the kinase inhibitors staurosporine and H7, but not by H8 or a peptide inhibitor of cAMP-dependent protein kinase. These results suggest that ADR activity is generated by the phosphorylation of a protein which is present in quiescent cells, and that the phosphorylation is not mediated by cAMP-dependent protein kinase. The potential involve-

ment of protein kinase C and several membrane-associated tyrosine kinases is currently being investigated.

DEFECTS IN ADR RESPONSE IN LYMPHOCYTES FROM AGED INDIVIDUALS

T lymphocytes from aged individuals are less responsive to mitogenic stimulation than lymphocytes from young control individuals [43,44]. A number of studies have suggested that these cells are defective in either IL-2 production or IL-2 responsiveness. As the generation of ADR activity is part of the IL-2 proliferative response, it was important to determine if changes in ADR production or response were associated with the proliferative defects seen in aged lymphocytes. Extracts were prepared from PHA-stimulated PBL from aged (66–72 years) and young adult (22–30 years) normal human donors and tested for ADR activity [45]. The extracts from aged donors contained as much ADR activity as extracts from young donors, even though the PHA responsiveness of lymphocytes from many (but not all) aged donors was significantly less than that of those from young donors. Cytoplasmic extracts from nonstimulated lymphocytes from both young and aged donors failed to induce DNA synthesis. These results show that the decreased proliferative response of lymphocytes from aged donors was not due to an inability to produce ADR activity.

The nuclei from resting lymphocytes of aged individuals with either intact or defective mitogen responsiveness were assayed for their ability to respond to exogenous ADR. Control nuclei preparations were derived from resting young lymphocytes. Nuclei derived from young lymphocytes and from lymphocytes from aged individuals with intact PHA responsiveness were comparable in their ability to incorporate ^{3}H-dTTP in response to ADR. However, nuclei from aged individuals with defective proliferative responses incorporated far less ^{3}H-dTTP in response to ADR. There was a clear correlation between the response of intact cells to PHA and the response of isolated nuclei from these cells to ADR. When plotted, the relationship between the ability of the intact cells to proliferate in response to PHA and the ability of the nuclei to respond to ADR is linear. These results indicate that the loss of nuclear responsiveness to ADR may be a major factor in the age-related loss of proliferative capacity seen in these lymphocytes.

QUIESCENT CELLS SYNTHESIZE AN INHIBITOR OF ADR ACTIVITY

Initial experiments with extracts prepared from quiescent cells demonstrated that they contained no ADR activity. Experiments from other laboratories indicated that nondividing fibroblasts contained an activity which could suppress DNA replication in nuclei from proliferating fibroblasts [46,47]. To determine if the quiescent extracts contained factors which modulated ADR activity, extracts prepared from unstimulated PBL were mixed with extracts prepared from stimulated PBL and MOLT 4 cells and assayed for ADR activity [30]. DNA synthesis was markedly suppressed in wells containing mixtures of extracts as compared to wells which contained an equal volume of the proliferating cell extract. This suppression was also observed with nuclei obtained from human PBL instead of frog spleen cells.

These results demonstrated that quiescent PBL contained an inhibitory activity towards ADR. Trypsin treatment of the quiescent extracts abolished the inhibition, indicating that the inhibitor was a protein. The inhibitory factor was stable at 4°C for at least 24 h and for one round of freeze-thawing. The inhibitory activity is heat-stable and is unaffected by a 20 min incubation at 56°C, conditions which destroy ADR activity. The inhibitory activity was completely retained by a XM50 membrane, suggesting that the inhibitory factor was ≥ 50 kD.

To establish a timecourse for the appearance of the inhibitory activity, cytoplasmic extracts were prepared from freshly isolated PBL and from these unstimulated PBL after various times of culture in resting medium. Inhibitory activity was not seen in extracts from freshly isolated PBL, but became detectable between 2 and 6 h of culture and reached maximal levels at 18 h. As freshly isolated PBL incorporated low levels of ^{3}H-dTTP, a property which diminished with time in culture, the initial absence and time-dependent onset of ADR inhibitory activity may be related to the state of quiescence.

To exclude the possibility that contaminating macrophages were a source of ADR inhibitory activity, extracts were prepared from unfractionated PBL and from macrophage-depleted PBL which had been cultured overnight in resting medium. Both extracts had identical ADR-inhibitory activity, indicating that the inhibitory factor was neither produced by macro-

phages nor produced by lymphocytes in response to macrophages or their products.

DEFECTS IN ADR INHIBITOR RESPONSE IN NEOPLASTIC CELLS

The inhibitor of ADR activity was defined by the ability to inhibit ADR-mediated initiation of DNA synthesis in quiescent nuclei from nontransformed cells. The next experiments tested whether the inhibitor could suppress ongoing DNA synthesis in nuclei from mitogen-activated PBL and transformed cell lines. Nuclei were prepared from PHA-treated lymphocytes and incubated with inhibitor-containing extracts from resting PBL [48]. Addition of the resting extract to nuclei that were actively synthesizing DNA resulted in a marked decrease in DNA synthesis, while extracts from activated cells supported ongoing DNA synthesis. Thus the inhibitor activity can suppress DNA replication in nuclei from proliferating normal cells.

Since several continuously proliferating neoplastic cell lines produce high levels of ADR, it was important to determine whether their nuclei responded to the ADR inhibitor. Nuclei from several of these cell lines, including MOLT-4, RPMI 8392, and BW 5147, were isolated and incubated in the presence or absence of cytoplasmic extracts from resting PBL. Nuclei from mitogen-stimulated PBL were also incubated in the presence or absence of the inhibitor as a control. As seen previously, resting cell extracts were capable of inhibiting DNA replication in nuclei from mitogen-activated PBL. However, these extracts had no effect on DNA replication in nuclei from the neoplastic cells. The inability of the neoplastic nucleus to respond to the ADR inhibitor could be a significant factor in the continuous proliferation of these cells.

The failure of these nuclei to respond to the ADR inhibitor derived from quiescent PBL raises the possibility that cells with this defective response may concurrently produce both ADR and the ADR inhibitor. We have recently identified such an ADR inhibitor in a subline of MOLT-4 cells which also produces high levels of ADR activity (Coffman et al., submitted). Like the PBL inhibitor, it is heat stable and inhibits ADR in a dose-dependent manner. It does not inhibit DNA polymerase activity. The inhibitor must be present at the initiation of DNA replication to be effective, and loses most of its effectiveness if it is added after replication has began. These results support the hypothesis that ADR is a true initiator of DNA replication and not simply a limiting factor in the replicative machinery, and that the ADR inhibitor inhibits initiation of DNA replication and not elongation.

SUMMARY AND CONCLUSIONS

In T lymphocytes, the initiation of DNA replication appears to be regulated by two distinct activities: one associated with proliferation which mediates initiation, and another associated with quiescence which blocks initiation. Activated lymphocytes and proliferating lymphoid cell lines produce an activity, termed ADR, which can initiate DNA replication in isolated quiescent nuclei. ADR is heat-labile, has protease activity or interacts closely with a protease, and is distinct from the DNA polymerases. ADR activity is absent in quiescent lymphocytes and appears in mitogen-stimulated lymphocytes after IL-2 binding. The generation of active ADR appears to be mediated by phosphorylation of a protein which is present in resting cells and not by synthesis of a new protein. Nuclei from mitogen-unresponsive lymphocytes fail to initiate DNA replication in response to ADR, a defect which appears to be an important factor in the age-related decline in immune function. Similar cytoplasmic activitors of proliferation from mature fibroblasts [24] as well as embryonic tissues [22,23] have been described; thus it is likely that parallel mechanisms are operating in other cell types.

Quiescent lymphocytes lack ADR and synthesize an ADR-inhibitory activity. The ADR inhibitor is a heat-stable protein which suppresses the initiation of DNA synthesis in isolated quiescent nuclei, but is ineffective at suppressing elongation once DNA strand replication has begun. Nuclei from several neoplastic cell lines fail to respond to the ADR inhibitor, which may be an important factor in the continuous proliferation of these cells. At least one of these neoplastic cell lines produces both ADR and an inhibitory factor, which blocks ADR activity in normal nuclei but is ineffective in regulating DNA replication in its own nucleus. These findings suggest that the regulation of proliferation is dependent on the balance between activating and inhibitory pathways, and that perturbation of either pathway may lead to abnormal patterns of growth.

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