Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7824–7828, August 1995
Cell Biology

The product of the ataxia–telangiectasia group D complementing gene, *ATDC*, interacts with a protein kinase C substrate and inhibitor

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Communicated by Ira Herskowitz, University of California, San Francisco, April 7, 1995 (received for review November 17, 1994)

ABSTRACT **Ataxia–telangiectasia (AT) is an autosomal recessive human genetic disease characterized by immunological, neurological, and developmental defects and an increased risk of cancer. Cells from individuals with AT show sensitivity to ionizing radiation, elevated recombination, cell cycle abnormalities, and aberrant cytoskeletal organization. The molecular basis of the defect is unknown. A candidate AT gene (*ATDC*) was isolated on the basis of its ability to complement the ionizing radiation sensitivity of AT group D fibroblasts. Whether *ATDC* is mutated in any AT patients is not known. We have found that the ATDC protein physically interacts with the intermediate-filament protein vimentin, which is a protein kinase C substrate and colocalizing protein, and with an inhibitor of protein kinase C, hPKCI-1. Indirect immunofluorescence analysis of cultured cells transfected with a plasmid encoding an epitope-tagged ATDC protein localizes the protein to vimentin filaments. We suggest that the ATDC and hPKCI-1 proteins may be components of a signal transduction pathway that is induced by ionizing radiation and mediated by protein kinase C.**

Cultured cells from ataxia–telangiectasia (AT) patients show increased sensitivity to ionizing radiation and reduced cell cycle delay in G_1, S, and G_2 phases in responses to ionizing radiation (reviewed in ref. 1). The failure to delay the cell cycle in S phase exhibited in fibroblasts obtained from patients with AT (2), termed radioresistant DNA synthesis, has been used to identify four complementation groups in cell fusion experiments (A, C, D, and E; refs. 3–5). Linkage analysis (6) and chromosome transfer experiments (7) have mapped the A, C, and D complementation groups to chromosome 11 region q22–23.

The search for a molecular defect in AT has revealed a wide variety of abnormal phenotypes. There is a decrease in the extent of error-free repair of chromosome damage in AT cell lines (8, 9), although no specific repair activity has been shown to be defective. The high frequency of chromosome translocations at the T-cell receptor and antibody gene loci and the sensitivity to ionizing radiation (10, 11) are consistent with a defect in the rejoining of double-strand breaks; however, the overall rate of double-strand break repair in AT is normal (1). Misrepair of plasmid strand breaks in cell extracts has been reported for AT cells (12); however, other AT cell lines do not show misrepair of such breaks (13). AT cell lines show a large increase in the frequency of homologous intrachromatid recombination (14).

AT cells show a delay in the ionizing radiation-induced elevation in the level of the p53 tumor-suppressor protein (15), suggesting that the AT product(s) may be involved in detection of DNA damage and/or transduction of a DNA damage signal. The AT-mediated induction of p53 is also blocked by inhibitors of protein kinase C (PKC) but not by inhibitors of cAMP-dependent protein kinase (16), indicating that AT and PKC may function together in this pathway.

There have been several attempts to clone AT gene(s) by functional complementation of AT cell lines[¶] (17, 18). The radiosensitivity of AT group D cell lines can be suppressed by a cosmid clone that maps to band q23 of chromosome 11 (17). Several mRNAs (1.8, 2.6, and 3.0 kb) are made from this locus (19). The cDNA of the 3.0-kb mRNA (*ATDC*) has been isolated (19) and characterized (20). Single-strand conformation polymorphism analysis has failed to detect mutations in the 3.0-kb cDNA from a group D cell line (20). Expression cloning of several other cDNAs that also suppress radiosensitivity of AT cell lines but do not map on chromosome 11 (ref. 18 and §) demonstrates that overexpression of non-AT genes can result in suppression of the radiosensitivity of AT cell lines.

Regardless of whether *ATDC* is a bona fide AT gene, it is likely that *ATDC*, as well as other genes that suppress radiosensitivity, functions in a pathway that is defective in AT. To learn more about the function of *ATDC*, we used the yeast two-hybrid system to identify cellular proteins that physically interact with the *ATDC* gene product. We report here that ATDC interacts with the intermediate-filament protein vimentin, which is a PKC substrate and colocalizing protein; with a small inhibitor of PKC, human PKC inhibitor 1 (hPKCI-1); and with the product of a vimentin-related cDNA. We suggest that the ATDC and hPKCI-1[‖] proteins may be components of a signal transduction pathway that is induced by ionizing radiation and mediated by PKC.

MATERIALS AND METHODS

Plasmids. pYZ45 is a 2μ-derived plasmid carrying ampicillin resistance (Ap^r) and *HIS3* markers and expressing a *lexA-ATDC* gene fusion under control of the *ADH1* promoter (*pADH::lexA-ATDC*; ref. 33); pYZ46 is similar but with a *pADH1*-promoted *lexA*-human lamin gene fusion. pBMT116 is a 2μ derivative with a *TRP1* marker and *pGAL1*-promoted *lexA* (S. Fields, State University of New York at Stony Brook). pEG202 is a 2μ derivative with a *HIS3* marker and *pADH1*-promoted *lexA* (21). pCTC53 is a 2μ derivative with a *TRP1* marker and a *pADH1*-promoted *lexA*-human lamin gene fusion (S. Fields). pQE16 (Qiagen) carries an Ap^r marker and allows

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Abbreviations: AT, ataxia–telangiectasia; FITC, fluorescein isothiocyanate; HA, hemagglutinin; NTA, nitrilotriacetic acid; PKC, protein kinase C; hPKCI-1, human PKC inhibitor 1; SV40, simian virus 40.

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¶Ziv, Y., Bar-Shira, A. & Shiloh, Y. (1994) Proceedings of the 6th International Ataxia-Telangiectsia Workshop, May 22–25 1994, Birmingham, England, p. 14 (abstr.).

‖The hPKCI-1 sequence has been deposited in the GenBank data base (accession no. U27143).

plac-promoted expression of cDNA inserts as proteins with hexahistidine (His_6) at the C terminus to facilitate purification; pYZ53 is pQE16 with an *ATDC* insert. In pCB621, transcription of the fusion encoding ATDC with the 9-aa influenza hemagglutinin (HA) epitope at the C terminus is controlled by the simian virus 40 (SV40) early promoter (*pSV40*); pCB622 is identical to pCB621 except that the *ATDC-HA* coding sequence is in the antisense orientation.

Cell Lines. LM217 is a normal human fibroblast cell line transformed with SV40 (19). MCF7 is a human breast cancer cell line that lacks vimentin filaments (22) due to a failure to express vimentin (23). LM217 was grown in DME-H21 medium supplemented with 10% fetal bovine serum, and MCF7 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Affinity Chromatography. The frozen cell pellet from 50 ml of an isopropyl β-D-thiogalactopyranoside-induced culture of *Escherichia coli* M15 (Qiagen) containing plasmid pYZ53 (*plac*::*ATDC*-His_6) was thawed and suspended in 1 ml of lysis buffer (50 mM sodium phosphate, pH 8.0/300 mM NaCl/20 mM imidazole/0.1% lysozyme/1 mM phenylmethanesulfonyl fluoride). The suspension was incubated on ice for 30 min, homogenized, and centrifuged at 10,000 × *g* for 15 min at 4°C; the supernatant was collected. A Ni^{2+}-nitrilotriacetic acid (NTA)-agarose spin column was preequilibrated with 600 μl of buffer I (50 mM sodium phosphate, pH 8.0/300 mM NaCl/20 mM imidazole) by centrifugation for 2 min at 2000 rpm at 4°C. Six hundred microliters of the cleared cell lysate was loaded onto the preequilibrated Ni^{2+}-NTA spin column by centrifugation in order to bind recombinant ATDC-His_6 to the column. The Ni^{2+}-NTA-ATDC column was washed twice with buffer II (50 mM sodium phosphate, pH 8.0/300 mM NaCl/20 mM imidazole/1 mM phenylmethanesulfonyl fluoride) by centrifugation. Then 350 μg of lysate from cell line LM217, equilibrated with buffer II in a final volume of 600 μl, was loaded onto the Ni-NTA-ATDC (or Ni^{2+}-NTA-only control) spin column. The flowthrough was collected. The Ni^{2+}-NTA-ATDC spin column was washed twice with 600 μl of buffer II and the washes were collected by centrifugation. Bound proteins were eluted twice with buffer III (50 mM sodium phosphate, pH 8.0/300 mM NaCl/250 mM imidazole). The eluted fractions were collected by centrifugation.

Indirect Immunofluorescence. pCB621 (*pSV40*::*ATDC-HA*) and control plasmid pCB622 (*pSV40*::antisense *ATDC-HA*) were transfected into fibroblast cell lines (Ca transfection system; BRL), and transfectants were grown on polyornithine-coated cover slips. After a 3-day incubation to allow phenotypic expression, the cells were fixed in cold acetone and immunostaining was performed (24) with either polyclonal or monoclonal anti-HA primary antibody and fluorescein isothiocyanate (FITC)-goat anti-rabbit antibody or Texas Red-conjugated goat anti-mouse secondary antibody (Caltag, South San Francisco, CA). Vimentin was detected with the monoclonal antibody V-9 (Sigma) and FITC-conjugated goat anti-mouse antibody (Caltag). Immunofluorescence was detected with a Zeiss Axioplan microscope and a ×63 or ×100 objective.

RESULTS

Vimentin and a PKC Inhibitor Interact with ATDC. To identify human genes encoding proteins that interact with ATDC, we used the yeast (*Saccharomyces cerevisiae*) two-hybrid system (25). The entire 588-aa open reading frame of *ATDC* was fused to a sequence encoding the N terminus of the sequence-specific DNA-binding protein LexA, and the fusion protein was expressed in a yeast strain containing both *LEU2* and *lacZ* reporter genes with LexA binding sites in their promoters (21). A library of HeLa cell cDNA fused to a sequence encoding the C-terminal end of a transcriptional activation domain was introduced into this yeast strain. ATDC-interacting proteins expressed from this library should activate the *LEU2* and *lacZ* reporters in the presence of the LexA-ATDC hybrid protein. Approximately 300,000 transformants (roughly three genome equivalents) were examined to obtain nine cDNAs that reproducibly showed reporter activation with *lexA-ATDC* but that did not show reporter activation with either *lexA*-bicoid (bicoid is a transcriptional regulatory protein from *Drosophila*) or *lexA*-human lamin controls. DNA sequences of these nine cDNAs were determined and examined for homology with known genes.

Seven of the nine cDNAs encoded fusions to the intermediate-filament protein vimentin. The fusions contain 245–308 of the 465 aa of vimentin and all include the C-terminal tail region which is implicated in lateral packing (26) and network formation (27) of vimentin filaments. Of the seven vimentin fusions, five are fused at different positions in the vimentin molecule, indicating that they were isolated independently.

A second ATDC-interacting protein is 96% identical to a bovine PKC inhibitor, PKCI-1 (Fig. 1 and ref. 28). We termed this human protein hPKCI-1. The bovine protein had been identified biochemically and its amino acid sequence was determined directly (28). The original hPKCI-1 clone from the two-hybrid cDNA library encoded only the C-terminal 80 aa of hPKCI-1. With PCR primers, a longer hPKCI-1 cDNA that contained an open reading frame spanning the entire bovine protein was obtained and sequenced. The great degree of conservation with the bovine protein suggests that the human protein is also a PKC inhibitor.

A third ATDC-interacting protein is identical to a previously identified gene product called HUMHMP4 (29). Our analysis with the BLITZ homology search program (EMBL version 1.5) indicates that HUMHMP4 is most closely related to the vimentin/desmin family of type III intermediate-filament proteins (Fig. 2). A region of 122 aa is 36% identical between vimentin (aa 105–226) and HUMHMP4 (aa 434–555). Quantitative β-galactosidase assays indicate that the relative strength of association of ATDC-interacting proteins with ATDC in yeast is, in increasing order of strength, vimentin (1×), hPKCI-1 (2×), and HUMHMP4 (4×).

ATDC and Vimentin Interact *in Vitro*. We purified recombinant ATDC protein from *E. coli* as an ATDC-His_6 fusion to determine whether ATDC and vimentin interact *in vitro*. *E. coli* extracts were made following induction of a *plac*::*ATDC*-His_6 construct (pYZ53) with IPTG and loaded onto a Ni^{2+}-NTA agarose column. The column was washed stringently so that ATDC was the only bound protein detectable by Coomassie blue staining. Then a cell extract

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human PKCI-1   MADEIAKAQV ARPGGDTIFG KIIRKEIPAK IIMEDDMCLA FHDISPQAPT  50
bovine PKCI-1  -ADEIAKAQV ARPGGDTIFG KIIRKEIPAK IIMEDDMCLA FHDISPQAPT  49

human PKCI-1   HFLVIPKKMI SQISMAEDDD ESLLGHLMIV GKKCAADLGL MKGYRMVVNE 100
bovine PKCI-1  HFLVIPKKMI SQISMAEDDD ESLLGHLMIV GKKCAADLGL MKGYRMVVNE  99

human PKCI-1   GSDGGQSVYH VHLHVLGGRQ MNWPPG                         126
bovine PKCI-1  GSDGGQSVYH VHLHVLGGRQ MNWPPG                         125
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FIG. 1. Homology between human and bovine PKCI-1 amino acid sequences.

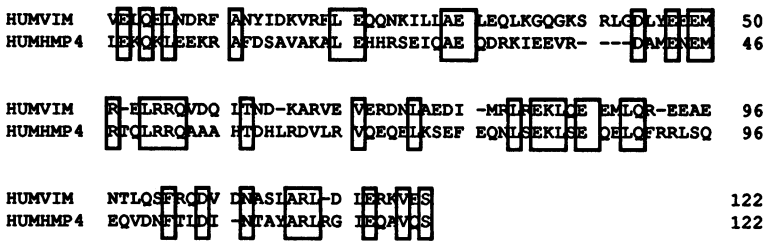

FIG. 2. Homology between HUMHMP4 and vimentin. The alignment was made with the protein homology search program BLITZ. The regions shown are aa 105–226 for human vimentin (HUMVIM) and aa 434–555 for HUMHMP4.

'from the wild-type human fibroblast line LM217 was loaded onto the Ni^{2+}-NTA agarose column containing bound ATDC. After three column washes, 99% of the loaded protein had flowed through the column as determined by protein assay. Bound proteins were eluted and analyzed by SDS/polyacrylamide gel electrophoresis and immunoblotting. Immunoblotting with an anti-vimentin monoclonal antibody demonstrated that about 50% of the vimentin in the crude extract bound to the column under conditions where 99% of all proteins do not bind (Fig. 3), indicating that vimentin and ATDC interact specifically. Control experiments indicated that vimentin did not bind to Ni^{2+}-NTA agarose that did not contain bound ATDC (data not shown).

ATDC Localizes to Cytoplasmic Filaments in Cultured Cells. For indirect immunofluorescence, a fusion was made between ATDC and the 9-aa HA epitope of influenza virus (30). The *ATDC-HA* fusion, expressed from the SV40 promoter, was transiently transfected into the SV40-transformed normal human fibroblast line LM217. After 3 days the cells were fixed and incubated first with a monoclonal antibody to the HA epitope and then with FITC-conjugated secondary antibody.

Examination of the transfected cells by fluorescence microscopy revealed that ATDC was localized to cytoplasmic filaments. Fig. 4 *Upper* shows the dense cytoplasmic filaments observed when the microscope was focused on a plane in the cytoplasm. Fig. 4 *Lower* shows the less dense staining seen over and around the nucleus in a different focal plane. Only about 1 in 10,000 cells stained positively, most likely due to the low transfection efficiency in these cells (17). We have stained transfected cells with both monoclonal and polyclonal antibodies to the HA epitope and observed essentially identical results (data not shown). Over 50 individual transfected cells were photographed and all showed a filamentous staining pattern. The staining observed for ATDC was very similar to vimentin staining (31). Untransfected control cells or cells that were transfected with a promotorless *ATDC-HA* gene did not give rise to any fluorescent cells. Examination of *ATDC-HA* expressing cells with a phase-contrast lense did not reveal any abnormal morphology of these cells. X-irradiation of transfected cells with up to 500 rads (1 rad = 0.01 Gy) did not affect ATDC localization for up to 2.5 hr after irradiation (data not shown).

ATDC Colocalizes with Vimentin in Cultured Cells. We performed a dual labeling experiment in which transfected fibroblasts were simultaneously stained with a polyclonal antibody to the HA epitope present on the ATDC-HA fusion and a monoclonal antibody to vimentin. The ATDC-HA antibody was detected with a Texas Red-conjugated goat

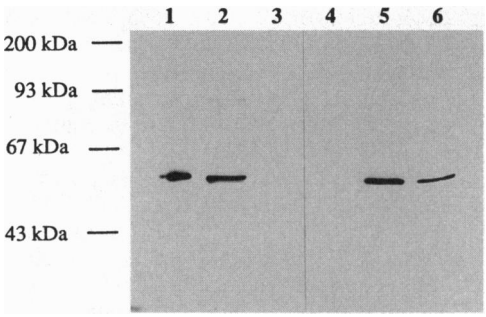

FIG. 3. Vimentin binds to an ATDC affinity column. ATDC-His_6 was bound to a Ni^{2+}-NTA-agarose. Whole cell extract of LM217 was loaded on the column. After the column was washed, proteins specifically bound to ATDC were eluted under denaturing conditions, fractionated in a polyacrylamide gel, blotted to nitrocellulose, and probed with an antibody against vimentin, and stained with horseradish peroxidase-conjugated secondary antibody and the ECL detection system (Amersham). Lane 1, crude cell extract from LM217; lane 2, flowthrough; lanes 3 and 4, washes I and II, respectively; lanes 5 and 6, elutions I and II, respectively. Ten microliters of each fraction (200-μl total fraction volume) was loaded on an SDS/13% polyacrylamide gel, except for the LM217 cell extract (lane 1), where 5 μl was loaded.

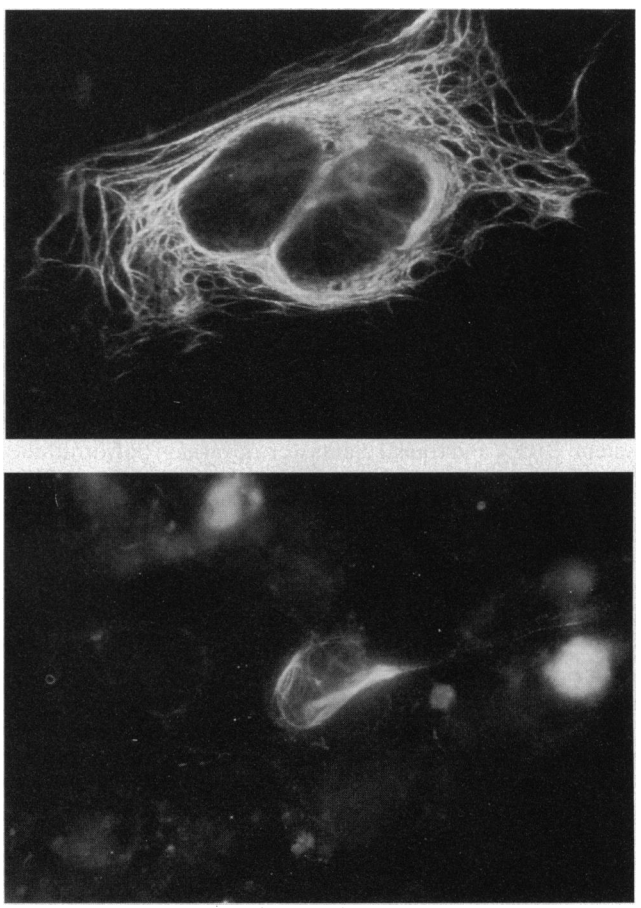

FIG. 4. ATDC localizes to cytoplasmic filaments in cultured cells. Two LM217 cells showing localization of ATDC to cytoplasmic filaments. (*Upper*) Focus on the plane of cytoplasmic filaments. (*Lower*) Focus on the plane of the nuclear periphery containing cytoplasmic filaments.

anti-rabbit antibody, and the monoclonal antibody to vimentin was detected with a FITC-conjugated goat anti-mouse antibody. A representative result from the dual labeling is shown in Fig. 5. ATDC staining (Fig. 5 *Upper*) is largely coincident with vimentin staining (Fig. 5 *Lower*) in the two cells shown, indicating that the two proteins colocalize in living cells. Control experiments demonstrated that cells stained only for ATDC-HA showed no FITC signal and cells stained only for vimentin showed no Texas Red signal (data not shown). In addition, neither secondary antibody alone showed a signal. We focused on the less dense filamentous staining observed over nuclei where the resolution of individual filaments is highest. In this region coincident staining was apparent (Fig. 5). However, in most cells, there were also regions of filamentous ATDC staining where vimentin staining was not observed. Furthermore, we have observed ATDC filaments in the vimentin-negative breast cancer cell line MCF7 (data not shown), indicating that ATDC does not solely colocalize with vimentin. Thus, ATDC localizes primarily, but not exclusively, to vimentin filaments in cultured cells.

DISCUSSION

We have found that the *ATDC* product, which complements the ionizing radiation sensitivity of AT group D cell lines, physically interacts with vimentin, a type III intermediate filament; with hPKCI-1, a small protein inhibitor of PKC; and with a vimentin-related cDNA product, HUMHMP4. Indirect immunofluorescence indicates that ATDC resides in the cytoplasm and colocalizes with intermediate filaments that are largely, but not exclusively, coincident with vimentin filaments. That ATDC can associate with nonvimentin filaments is supported by the observations that localization to cytoplasmic filaments is observed in MCF7 cells, which entirely lack vimentin expression, and that ATDC also interacts with a vimentin-related product, HUMHMP4, in the two-hybrid system.

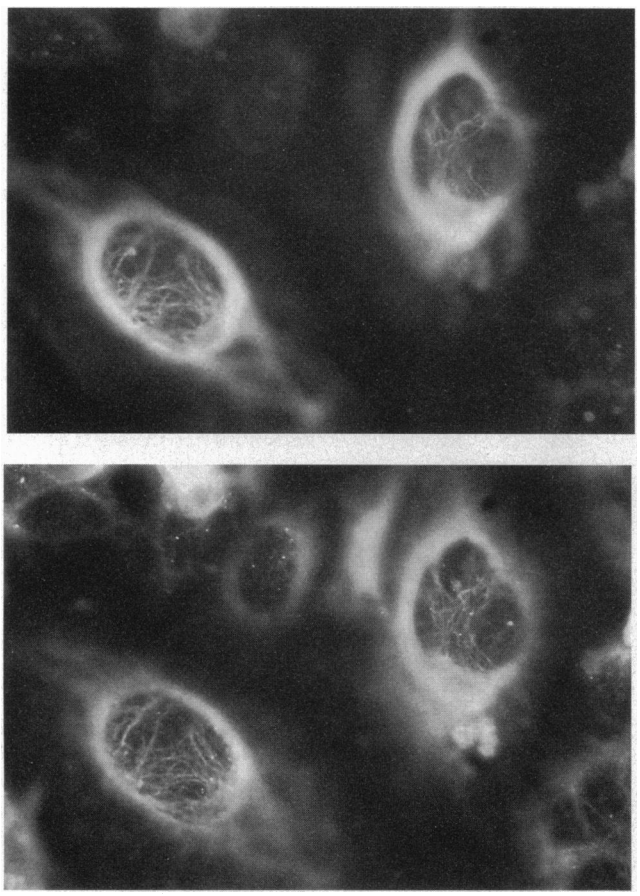

FIG. 5. Colocalization of ATDC and vimentin in LM217 cells by dual labeling indirect immunofluorescence. (*Upper*) ATDC localization was performed with a primary polyclonal antibody to the HA epitope (12CA5) and a secondary antibody conjugated to Texas Red. (*Lower*) Vimentin was localized by use of a monoclonal antibody and FITC-conjugated goat anti-mouse antibody.

The molecular basis of the defect in AT is not known, although the diversity of phenotypes has led to the suggestion that a signaling mechanism may be defective (2, 15, 32). This explanation is supported by the finding that the ionizing radiation-induced increase in the p53 protein is both delayed and reduced in extent in AT cell lines (15, 16). PKC is also required for p53 increase, as specific PKC inhibitors block the increase in p53 following ionizing radiation (16), indicating that AT, PKC, and p53 function in a common pathway. Recent experiments suggest that aberrant p53 function may be a major factor in the hypersensitivity of AT cells to ionizing radiation (32). Furthermore, it has been suggested that one function of intermediate filaments is to mediate intracellular signaling by PKCs, since PKCβ colocalizes with vimentin (34). ATDC complements ionizing radiation sensitivity in AT cell lines and, as we show in this report, interacts with components of a PKC pathway. Taken together, these data suggest a model in which ATDC, hPKCI-1, and vimentin are components of an ionizing radiation-induced signal transduction pathway that is defective in AT. In this model, the pleiotropic phenotype of AT would result from the failure of downstream events in a PKC-mediated signal transduction cascade. For example, ATDC may activate a PKC isozyme by complexing hPKCI-1 following exposure to ionizing radiation. Which of the 10 PKC isozymes (35) is involved is not known, although PKC-β has been shown to colocalize with vimentin (34). However, PKC-ε has been shown to be induced by ionizing radiation (36) and recent experiments indicate that PKC-ε is much less abundant in AT cell lines (37). Hallahan *et al.* (38) have demonstrated that the ionizing radiation-induced signal required for transcriptional induction of the tumor necrosis factor α gene involves the activation of phospholipase A_2 at the cell membrane and, subsequently, PKC activity. Interestingly, overexpression of phospholipase A_2 has also been shown to complement partially the ionizing radiation sensitivity of AT group D cell lines[§].

An alternative model is that ATDC complements the ionizing-radiation sensitivity of an AT group D cell line by virtue of its ability to stabilize defective cytoskeletal filaments that may arise in AT cells. AT cell lines display aberrantly aggregated actin filament arrays (39), although aberrant intermediate filaments have not been reported. ATDC displays several regions of homology to vimentin, including a sequence that resembles the highly conserved rod domain sequence TYRKLLEGE (TYHVLLEGE in ATDC) that is critical for proper filament assembly in the cell (27). Other regions of similarity exist between ATDC and vimentin and between ATDC and trichohyalin (40), a vimentin-associated protein. However, these regions are in the putative leucine zipper of ATDC, making their significance unclear. The precise *in vivo* functions of intermediate filaments remain obscure (26) but are thought to include maintenance of cellular mechanical stability (26) and, perhaps, intracellular signaling (34, 41). Mutations in intermediate filaments give rise to several genetic diseases. For example, human epidermolysis bullosa simplex is caused by keratin mutations (26) and results in keratinocytes with a defective mechanical framework. In addition, a mouse defect similar to amyotrophic lateral sclerosis (ALS) can be caused by a neurofilament NF-L mutation (42). ALS, like AT, is a degenerative brain disease. The purification of recombinant ATDC will allow direct testing of whether ATDC forms filaments *in vitro* and whether ATDC interaction with hPKCI-1 leads to PKC activation.

Note Added in Proof. Recently a gene has been identified that is mutated in AT patients from all complementation groups (43). The gene, termed ATM, encodes a protein with a putative phosphatidylinositol 3-kinase domain. The fact that ATM encodes a putative lipid-mediated signalling molecule is consistent with a model in which ATDC and PKC function downstream from ATM in this pathway.

We thank Roger Brent for providing the yeast strains and plasmids for the two-hybrid *LEU2* selection system, William Dewey and members of our laboratories for critical comments on the manuscript, and Pragati Bakshi for help in preparing the manuscript. We thank Joe Gray for the use of his fluorescence microscopes, Charlie Vidair for providing cell line MCF7, and Christine Weber for providing plasmid pcD2E. This work was supported by a grant to M.F.C. from the AT Children's Project.

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