Communication

Cullin 4A Associates with the UV-damaged DNA-binding Protein DDB*

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Pavel Shiyanov, Alo Nag, and Pradip Raychaudhuri‡

From the Department of Biochemistry and Molecular Biology (M/C 536), University of Illinois, Chicago, Illinois 60612

The damaged DNA-binding protein (DDB) is believed to be involved in DNA repair, and it has been linked to the repair deficiency disease xeroderma pigmentosum. DDB also exhibits transcriptional activities. DDB binds to the activation domain of E2F1 and stimulates E2F1-activated transcription. Here we provide evidence that DDB or DDB-associated proteins are targets of cullin 4A (CUL-4A). CUL-4A is a member of the cullin family of proteins, which are believed to be ubiquitin-protein isopeptide ligases (type E3). The CUL-4A gene has been shown to be amplified and up-regulated in breast carcinomas. In this study, we identify CUL-4A as one of the DDB-associated proteins. CUL-4A co-immunoprecipitates with DDB, but not with a naturally occurring mutant of DDB. Moreover, CUL-4A in HeLa nuclear extracts co-purifies with DDB, suggesting they are parts of the same complex. The observation provides insights about CUL-4A, through an interaction with DDB, might be playing a role in the development of breast carcinomas.

Two mutants, 2RO and 82TO, have been characterized from XP-E patients. These mutants harbor single amino acid substitutions, R273H (2RO) and K244E (82TO), in the WD motif of p48 (DDB2 gene product) (10). These mutant p48 proteins are impaired in their ability to cooperate with the p125 subunit in damaged DNA binding assays (4, 10). The mutant 2RO is also incapable of forming a stable complex with the p125 subunit (11). p48 plays an important role in the nuclear localization of p125 (11). These two XP-E mutants of p48 are deficient in their ability to enhance nuclear localization of p125 (11).

DDB also possesses a transcriptional function (11–13). It can function as a transcriptional partner of E2F1, DDB associates with the C-terminal activation domain of E2F1 and cooperates with E2F1 to stimulate transcription from an E2F1-regulated promoter (13). Moreover, expression of DDB can overcome retinoblastoma inhibition of the E2F1-activated transcription (13). The transcriptional function depends upon both p48 and p125 subunits. The mutants 2RO and 82TO exhibit a deficiency in their transcriptional function (11). The p125 subunit of DDB has been shown to associate with several viral and cellular proteins. For example, p125 has been shown to bind the hepatitis B virus X protein, which is a potent activator of transcription (14). p125 also interacts with the V proteins encoded by paramyxovirus SV5, mumps virus, human parainfluenza virus, and measles virus (15). A recent study indicated an interaction between p125 and the C-terminal cytosolic region of the Alzheimer’s precursor protein (16). While the significance of many of these interactions is yet to be determined, the functional interaction between DDB and E2F1 suggests a role for DDB in the cell cycle.

CUL-4A is a member of the cullin family of proteins that are believed to be regulators of the cell cycle (17, 18). The members of the cullin family possess extensive sequence homology among each other and, therefore, are believed to have similar biochemical function (17, 18). CUL-1, the most well characterized cullin, was shown to be involved in cell cycle exit in Caenorhabditis elegans (17). The yeast homologue of CUL-1, cdc53, has been shown to be involved in proteolysis of the cell cycle inhibitor Sic1p and the G1 cyclins through the ubiquitin-proteasome pathway (19, 20). It has been proposed that CUL-1 and the other members of the cullin family act as E3 ligases, which are involved in selecting specific targets for ubiquitination (21). This notion is consistent with the observation that the cullins associate with other proteins involved in the ubiquitination of target proteins (21). Targets for the human cullins are not known. It has been shown that CUL-1 associates with the cell cycle regulatory protein cyclin A through an interaction with SKP1 (22). Here, we show that CUL-4A remains endogenously associated with DDB, suggesting the possibility that CUL-4A targets DDB or a DDB-associated protein involved in cell cycle regulation or DNA repair.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and DNA Transfection—Human osteosarcoma U2OS cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 5% CO2. Spinner cultures of HeLa cells were grown in minimum essential medium containing 5% calf serum. Plasmids used in this study have been described previously (11). DNA transfection was performed by the calcium-phosphate precipitation procedure as described previously (13).

Immunoprecipitation and Western Blots—The immunoprecipitation and Western blot experiments were carried out following previously

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‡ To whom correspondence should be addressed. Tel.: 312-413-0255; Fax: 312-413-0964; E-mail: pradip@uic.edu.

1 The abbreviations used are: DDB, damaged DNA-binding protein; XP-E, xeroderma pigmentosum group E; E3, ubiquitin-protein isopeptide ligase; HPLC, high performance liquid chromatography.
RESULTS

In an attempt to understand the function of DDB, we looked for cellular proteins that interact with the p48 subunit of DDB. Plasmids expressing T7 epitope-tagged p48 or the naturally occurring mutants of p48 (82TO and 2RO) were transfected into U2OS cells. The extracts of the transfected or mock-transfected cells were subjected to immunoprecipitation with a monoclonal antibody against T7 that is covalently linked to Sepharose beads. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by silver staining. We detected several polypeptides co-immunoprecipitating with the T7 antibody from the extracts of cells expressing the T7-p48 proteins, but not from the mock-transfected cells (Fig. 1). The bands migrating just below the 55-kDa marker corresponded to the wild-type or the mutant p48 proteins, as they were the only bands recognized by the T7 antibody in Western blots (not shown). The band migrating slightly above the 116-kDa marker corresponded to p125, as that was the only band detected by the p125 antibody in Western blot (not shown). As expected, the p48 transgene product co-immunoprecipitated p125, and the pattern of p125 co-precipitation with the wild-type and the mutant p48 was consistent with our previous observation in showing that the mutant 2RO failed to bind p125 (11).

There were several polypeptides of molecular mass ranging between 55 and 70 kDa specifically co-immunoprecipitated with DDB from transfected cells (Fig. 1). These polypeptides were co-immunoprecipitated from cells transfected with both wild-type and mutant p48. Interestingly, a polypeptide of about 80–85 kDa was co-immunoprecipitated specifically with the wild-type p48 and 82TO, but not with 2RO (marked with an arrow, Fig. 1). The overall band intensity in the lane for 82TO was low, but a darker stain did detect the 80–85-kDa polypeptide in that lane (not shown). To identify the polypeptides, the gel bands from a Coomassie Blue-stained gel were excised and subjected to trypsin digestion and sequence analysis of DDB-binding proteins—Trypsin digestion and sequence analysis of DDB-binding proteins—Trypsin digestion of gel bands containing DDB-binding proteins was performed in Harvard Microchemistry Facility (Cambridge, MA). The sequence analyses of the tryptic peptides were carried out in the same facility by microcapillary reverse-phase HPLC tandem mass spectrometry.

Peptide Antiserum—A chemically synthesized peptide, with the sequence ERDKDNPQXHVVAY, corresponding to human CUL-4A was conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce). The conjugate was used for rabbit immunization and antiserum production.

HeLa Nuclear Extracts and Heparin-Agarose Fractionation—HeLa nuclear extracts were prepared following the procedure of Dignam et al. (23). The heparin-agarose fractionation was carried out essentially following a previously described procedure (13).

DNA Affinity Chromatography—One milligram of sonicated salmon sperm DNA or sonicated salmon sperm DNA that was irradiated with 2 J/cm² of UV light in a Stratalinker (Stratagene) was linked to 4 ml of CNBr-activated Sepharose 4B following the procedure of Kodanaga et al. (24). Columns containing 0.5 ml of the affinity beads were used for chromatography of the heparin-agarose-purified DDB. The columns were equilibrated in buffer A (20 mM Hepes, pH 7.9, 0.2 mM dithiothreitol, 5% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) containing 0.1 M KCl. The heparin-agarose fraction was dialyzed against an excess of buffer A containing 0.3M KCl (3.2 ml) for 4 h. The dialyzed material was incubated with 5 μg/ml of sonicated salmon sperm DNA and then applied three times onto the affinity columns. After loading, the columns were washed with 4 ml of buffer A containing 0.1 M KCl followed by elution with buffer A containing 0.3 M KCl (3.2 ml) and 0.7 M KCl (3.2 ml).

RESULTS

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The blot was probed with the CUL-4A antibody and the lower part with T7 antibody. The T7 antibody detected the expression of the p48 transgene products. The wild-type and the two mutants, 82TO and 2RO, were expressed at approximately similar levels (Fig. 3). Consistent with the experiment in Fig. 1, both wild-type and 82TO co-immunoprecipitated CUL-4A, whereas 2RO failed to co-immunoprecipitate CUL-4A (Fig. 3, upper panel). This result also confirmed the observation that DDB associates with CUL-4A.

The interaction with CUL-4A could be an artifact of overexpression of the DDB proteins. For example, CUL-4A might target only misfolded DDB for proteolysis. Therefore, we investigated to ascertain an interaction between the endogenous gene products. HeLa cell nuclear extracts were fractionated to see whether CUL-4A co-purifies with DDB. HeLa nuclear extracts were first fractionated by heparin-agarose as described under “Experimental Procedures.” Briefly, extracts were applied onto the column at 0.1 M KCl. After loading, the column was successively washed with 3 bed volumes of buffer containing 0.25 M KCl and 0.25 M KCl. The column was finally eluted with a linear gradient (10 bed volumes) of KCl from 0.25 to 0.75 M. The column fractions were analyzed for the DDB proteins and CUL-4A by Western blot assays. The bands corresponding to p125, p48, and CUL-4A are indicated.

To further investigate the co-purification of CUL-4A with
DDB, we employed an affinity column specific for UV-damaged DNA-binding proteins. DDB was shown to possess high affinity for UV-damaged DNA, and it was purified using UV-damaged DNA affinity column (1). The heparin-agarose fractions containing the two DDB polyepitopes and CUL-4A were pooled and dialyzed as described under “Experimental Procedures.” The dialyzed material (1 mg/ml) was incubated with 5 μg/ml sonicated salmon sperm DNA. The material was then divided in two parts, and approximately equal amounts of the material were loaded onto affinity columns containing either double-stranded DNA-Sepharose or UV-damaged DNA-Sepharose. The material containing the salmon sperm DNA was loaded by gravity flow. The flow-through materials were collected as 0.1 stranded DNA-Sepharose or UV-damaged DNA-Sepharose. The material was then divided of p125.

This 170-kDa band might also represent a modified form marked by an asterisk, recognized by the p125 antibody co-purifying with p125. It is possible that this polypeptide corresponds to a p125-related protein that binds to UV-damaged DNA. This 170-kDa band might also represent a modified form of p125.

**DISCUSSION**

Results presented here suggest that CUL-4A has a high affinity for DDB in mammalian cells, and a significant part of CUL-4A remains associated with DDB in HeLa nuclear extracts. CUL-4A is mainly a nuclear protein, as only a small part (about 20%) is detected in the cytosolic extracts (data not shown). As can be seen in Fig. 4, greater than 50% of CUL-4A co-purified with DDB through a damaged DNA affinity column, suggesting that DDB is one of the primary targets of CUL-4A. CUL-4A has been shown to be amplified and up-regulated in breast cancers (26), and therefore, it is interesting that it targets DDB that has also been implicated in tumorigenesis. For example, a mutation in the p53 gene that leads to an inactivation of DDB function correlates with the development of skin cancer (25). Based on its homology with other cullins, CUL-4A is believed to be an E3 ligase involved in the ubiquitination of target proteins (21). (However, a direct evidence that CUL-4A possesses ubiquitin-ligase activity is yet to be seen.) It is possible that a key function of CUL-4A is to target DDB for proteolysis by the ubiquitin-proteasome pathway. In this scenario, a high level of CUL-4A expression (as in many breast cancers) would efficiently reduce the cellular levels of the DDB, accomplishing a result similar to that observed in DDB mutation.

DDB may not be the ubiquitination target of CUL-4A. For example, CUL-1, which is believed to be involved in ubiquitination and degradation of cyclin A, interacts with cyclin A indirectly through SKP1 (22). Therefore, it is also possible that CUL-4A targets a DDB-associated protein such as E2F1. It has been shown that E2F1 is degraded by the ubiquitin-proteasome pathway (27). Similarly, proteins that associate with DDB in its DNA repair pathway may also be targets of CUL-4A. We speculate that loss of DDB or loss of other components in the pathway of DDB function would have similar effect.

Surprisingly, we observed that a complex of CUL-4A and DDB could bind UV-damaged DNA. DDB has a high affinity for UV-damaged DNA and is believed to be involved in DNA damage recognition (5). Microinjection of DDB in repair-deficient XP-E cells can complement the deficiency, implying a role of DDB in DNA repair (9). The fact that CUL-4A remains associated with DDB and is able to interact with DDB bound to damaged DNA suggests a possible role for CUL-4A in DNA damage recognition. Mdm2, which is an E3 ligase involved in the ubiquitination of p53, also possesses a transcriptional activity (28, 29). Mdm2 was shown to have a transcriptional repression domain, which when tethered to a promoter can inhibit transcription (30). Thus an E3 ligase can have dual function. In this regard, it will be interesting to determine whether CUL-4A also possesses a role in damaged DNA recognition and repair.

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