Human DNA helicase VIII: a DNA and RNA helicase corresponding to the G3BP protein, an element of the Ras transduction pathway

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ABSTRACT

Human DNA helicase VIII (HDH VIII) was isolated in the course of a systematic study of the DNA unwinding enzymes present in human cells. From a HeLa cell nuclear extract a protein with an M<sub>r</sub> of 68 kDa in SDS–PAGE was isolated, characterised and microsequenced. The enzyme shows ATP- and Mg<sup>2+</sup>-dependent activity is not stimulated by RPA, prefers partially unwound 3′-tailed substrates and moves along the bound strand in the 5′ to 3′ direction. HDH VIII can also unwind partial RNA/DNA and RNA/RNA duplexes. Microsequencing of the polypeptide showed that this enzyme corresponds to G3BP, an element of the Ras pathway which binds specifically to the GTPase-activating protein. HDH VIII/G3BP is analogous to the heterogeneous nuclear ribonucleoproteins and contains a sequence rich in RGG boxes similar to the C-terminal domain of HDH IV/nucleolin, another DNA and RNA helicase.

INTRODUCTION

DNA helicases play a crucial role in DNA replication, repair and recombination (1); similarly RNA/RNA and RNA/DNA helicases have been identified and found to be essential in such functions as transcription, translation and RNA splicing (2,3), processes containing a sequence rich in RGG boxes similar to the C-terminal domain of HDH IV/nucleolin, another DNA and RNA helicase.

so-called ‘helicase domains’ has been obtained so far, there is no guarantee that they may represent specific recognition features for all duplex unwinding enzymes. In fact, in the course of our study, we have already identified two non-canonical helicases, namely human DNA helicases II and IV (HDH II and IV), which turned out to be respectively Ku (8) and nucleolin (9,10). Now, in the present report, we describe the identification and isolation of still another molecule that can unwind DNA and RNA partial duplexes in an ATP-dependent fashion, while lacking the canonical domains and presenting some similarity with the C-terminal portion of nucleolin; this molecule turned out to correspond to a protein identified in a different context and for which no evidence for helicase activity had been reported previously.

MATERIALS AND METHODS

Buffers

All the buffers used during the purification of HDH VIII contained 1 mM DTT, 1 mM sodium bisulfite, 0.5 mM PMSF, 1 µM pepstatin, 1 µM leupeptin. Buffer A contained 20 mM HEPES (pH 8.0), 0.1 mM NaCl, 1 mM EDTA and 20% glycerol. Buffer B contained 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 1 mM EDTA and 10% glycerol. Buffer C was the same as buffer B but with 0.1 M KCl. Buffer D contained 50 mM Tris–HCl (pH 8.0), 50 mM KCl and 1 mM MgCl<sub>2</sub>. The buffer for Mono Q column contained 50 mM Tris–HCl (pH 8.0), 150 mM KCl, 1 mM EDTA and 10% glycerol.

Preparation of DNA helicase substrates

The DNA substrates used in the helicase assay consisted of four different 32P-labelled oligonucleotides annealed to M13mp19 phage single-stranded (ss) DNA to create a partial duplex. Substrate without hanging tails: GTAAAACGACGGCCAGT, complementary to nt 6291–6307 of M13mp19 ssDNA; substrate with the same oligonucleotide carrying a hanging tail of 15 T residues at its 5′ end; substrate with the same hanging tail at its 3′ end; substrate with hanging tails at both the 5′ and 3′ ends. Aliquots of 25 ng of each oligonucleotide, labelled at the 5′ end with T4 polynucleotide Kinase (Promega Corporation, Madison, WI) and 0.9 MBq of [γ-32P]ATP (Amersham Life Sciences, UK), were subsequently annealed to M13mp19 phage ssDNA (4 µg) in 40 mM Tris–HCl (pH 8.0), 10 mM MgCl<sub>2</sub>,

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1 mM DTT and 50 mM NaCl. The mixture was heated at 95°C for 2 min, and slowly cooled to room temperature. Each substrate was purified by gel filtration through a 5 ml Sepharose 4B column.

Preparation of direction-specific substrates

Two different substrates both based on M13mp19 were prepared to determine the direction of unwinding of HDH VIII. The substrate used to detect an enzyme with a 5′ to 3′ polarity was annealed to a 33mer oligonucleotide (CTCTAGAGGTATCCCCGGTTACCGAGCTCGAATT) labelled at the 3′ end; the one used to detect an enzyme translocating in the 3′ to 5′ direction was annealed to a 3′ end-labelled 32mer oligonucleotide (CGACTCTAGAGATCCCCGGTTACCGAGCTCG). Preparation of the latter substrate followed the procedure described in the previous paragraph, whereas for the substrate with the 3′ end label, 25 ng of oligo were first annealed to M13 ssDNA (4 μg) and then labelled by adding to the 3′-OH end one 32P-labelled C residue with the DNA polymerase large fragment (New England Biolabs Inc., Beverly, MA). After slow cooling to room temperature the two substrates were each digested with Smal restriction enzyme (Promega) to generate a linearised molecule carrying a labelled 18mer oligonucleotide annealed to either end of M13 DNA. The reaction mixture in each case was passed through a 5 ml Sepharose 4B column to remove unreacted nucleotides.

Preparation of RNA/RNA and RNA/DNA substrates

The RNA/RNA and RNA/DNA substrates were obtained as follows: pGEM vector (Promega) containing the Ku 70 gene (11) was linearised by EcoRI and transcribed in vitro with the SP6 RNA polymerase from the specific promoter yielding a 1.3 kb RNA. A 17 bp DNA or RNA oligonucleotide complementary to the same region of Ku 70 (nt 1840–1856 of the Ku sequence) was synthesised and labelled in the 5′ end by T4 polynucleotide kinase (Promega) with 0.9 MBq [32P]ATP. A large excess of labelled oligonucleotide was mixed with 1 μM of synthesised Ku RNA, heated at 95°C for 2 min and allowed to anneal by slow cooling at room temperature. The substrates were then purified by gel filtration through a 5 ml Sepharose 4B column.

DNA helicase assay

The helicase assay measures the unwinding of a 32P-labelled DNA fragment from a partial duplex DNA molecule. The 10 μl reaction mixture contained 20 mM Tris–HCl (pH 9.0), 8 mM DTT, 2 mM MgCl2, 2 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 μg/ml BSA and 32P-labelled helicase substrate (1000 c.p.m.). The helicase fraction to be assayed was added to the mixture, incubated at 37°C for 30 min and the reaction was terminated by the addition of 0.3% SDS, 10 mM EDTA, 5% glycerol and 0.1% Bromophenol Blue. The products of the reaction were fractionated by electrophoresis on a 12% non-denaturing polyacrylamide gel. The gel was dried and the extent of DNA unwinding was quantitated by electronic autoradiography (Instant Imager, Packard Corp., Meriden, CT). One unit of DNA helicase is defined as the amount of enzyme unwinding 1% of the substrate in 1 min at 37°C (30% in the linear range of enzyme concentration dependence.

Affinity labelling with ATP

Affinity labelling with [α-32P]ATP by UV cross-linking, to reveal ATP binding by helicase, was performed as already described (8). A negative control containing all components of the reaction mixture but not irradiated with UV light was included to monitor effective binding and cross-linking of the cofactor to the enzyme. After the SDS–PAGE analysis of both samples, the gel was washed for 18 h with 10% acetic acid to remove non-bound radioactivity and then dried and exposed for autoradiography.

RESULTS

Purification of HDH VIII

The nuclear extract was obtained from 300 g of frozen HeLa cells by the procedure described by Dignam et al. (12) and the proteins with helicase activity were purified following the protocol of Tuteja et al. up to Fraction IV (13). Briefly, HeLa nuclear extract was precipitated by slowly adding ammonium sulfate (0.35 g/ml) with constant stirring. The mixture was stirred for an additional hour and the precipitated proteins were collected by centrifugation at 25 000 g for 30 min in a Sorvall ss34 rotor, dialysed in buffer A (Fraction I) and applied onto a Bio-Rex column (2.5 cm diameter × 33 cm) equilibrated with buffer A. The column was washed with 3 column vol of buffer A and the non-adsorbed protein fractions containing helicase activity were pooled (Fraction II). This pool was again treated with ammonium sulfate as described above and the resulting pellet, after centrifugation, was resuspended and dialysed in buffer B (Fraction III). This fraction was loaded onto a 1.6 cm × 14 cm DEAE-Sephacel column equilibrated with buffer B. The column was then washed with 5 column vol of buffer B and eluted with a 5 column vol linear gradient between 0.05 and 0.65 M KCl. The active fractions were pooled (Fraction IV). Fraction IV was loaded onto a heparin Sepharose column (1.4 × 4 cm) equilibrated with buffer C. The column was washed with 3 column vol of buffer C and subsequently eluted with a 5 column vol linear gradient from 0.1 to 1 M KCl in buffer C. The active fractions were pooled and diluted to 0.15 M KCl in buffer B (Fraction V). Fraction V was loaded onto an ssDNA column (ssDNA-D8273 Sigma, 1.6 × 3cm) equilibrated in buffer D. The column was then washed with 3 vol of buffer D and subsequently eluted with 5 vol of a gradient between 0.15 and 0.6 M KCl. The protein fractions eluting at 0.4 M salt contained helicase VIII (Fraction VI), whereas the fractions eluting at lower and higher ionic strength contained other unwinding activities that will be object of future studies. Fraction VI was loaded onto an FPLC monoQ column (Pharmacia-LKB, Upssala, Sweden) equilibrated with buffer B and the proteins were eluted with 10 column vol of a linear gradient from 0.15 to 1 M KCl; in the early fractions of the gradient two peaks of unwinding activity were visible that will also be the object of future investigations, whereas helicase VIII was eluted around 0.3 M KCl. The relevant fractions were pooled and concentrated by a second passage through the monoQ column (Fraction VII). The active fractions were analysed by SDS–PAGE and the silver-stained gel showed a prominent band of 68 kDa (Fig. 1). Table 1 summarises the purification procedure. As indicated in the table, and as previously reported (6,7,13), the early fractions do not allow a precise quantitation of the catalytic activity because of the presence of interfering activities, such as nucleases and phosphatases, as well as other helicasens; only after ssDNA Sepharose chromatography three distinct peaks of
activity can be identified in the eluate, as mentioned above. The pool eluting at 0.4 M was further purified on a Mono-Q column yielding still three different molecular species with helicase activity, whereof the most abundant corresponded to helicase VIII. This multiplicity of still unidentified helicases justifies the relative low recoveries of unwinding activity recorded for fractions VI and VII. Work is still in progress to identify the active molecules present in the other peaks fractionated away by the ssDNA and monoQ chromatographies.

Table 1. Purification of human DNA helicase VIII

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total proteins (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
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</thead>
<tbody>
<tr>
<td>Nuclear extract</td>
<td>470</td>
<td>2,620</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fract. I (Ammon. Sulfate ppt.)</td>
<td>250</td>
<td>2,150</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fract. II (Bio-Rex 70)</td>
<td>625</td>
<td>750</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fract. III (Ammon. Sulfate ppt.)</td>
<td>100</td>
<td>650</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fract. IV (DEAE Sephadex)</td>
<td>50</td>
<td>175</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fract. V (Heparin Sepharose)</td>
<td>50</td>
<td>40</td>
<td>66,000</td>
<td>1,650</td>
</tr>
<tr>
<td>Fract. VI (ssDNA Sepharose)</td>
<td>10</td>
<td>4</td>
<td>16,700</td>
<td>4,200</td>
</tr>
<tr>
<td>Fract. VII (MonoQ)</td>
<td>2</td>
<td>0.1</td>
<td>3,000</td>
<td>30,000</td>
</tr>
</tbody>
</table>

**Reaction requirements and preferred substrates**

No unwinding activity was detected in the absence of ATP or Mg$^{2+}$ (Fig. 2). The optimum concentration of ATP for helicase activity is ~3 mM, whereas ATP concentrations >15 mM inhibit the enzyme activity. The affinity of HDH VIII for ATP is further demonstrated by the cross-linking of this molecule to the enzyme operated by a mild UV irradiation (Fig. 3). The optimum concentration of Mg$^{2+}$ for the helicase activity was ~2 mM.

DNA unwinding by HDH VIII was assayed with different types of substrates; the results summarised in Figure 4 show that this enzyme unwinds preferentially a partial duplex substrate having a 17 bp annealed portion and a hanging 3′ tail: in fact this structure is unwound with the same efficiency as a structure having the same annealed portion but also hanging tails at both the 5′ and 3′ ends; conversely, a structure with the same annealed portion but no hanging tails is unwound with only half the efficiency of the other two substrates, and with the same efficiency as a similar substrate having a 5′ hanging tail. Thus the helicase seems to operate preferentially on a fork-like structure in which a relatively short 3′ single-stranded portion is present. Moreover HDH VIII failed to unwind a substrate with a 40 nt long annealed section.

**Direction of unwinding**

In order to determine the polarity of unwinding of HDH VIII we prepared two different substrates (Fig. 5) consisting of long linear ssDNA with short duplex ends on either side. The substrate which reveals the 3′ to 5′ direction was radioactively labelled at the 5′ end of the 18mer annealed at the 5′ end of the long ssDNA, whereas the substrate for the 5′ to 3′ direction was labelled at the 3′ end of an 18mer annealed at the 3′ end of the ssDNA. HDH VIII appears to unwind unidirectionally by moving in the 5′ to 3′ direction along the bound ssDNA (Fig. 5).

**Microsequencing**

Approximately 20 µg of protein were loaded on SDS–PAGE and, after migration, the Coomassie stained slide of the gel containing the protein was eluted and the latter was trypsinised and fractionated on an HPLC column. The four fragments obtained were microsequenced at their N-terminal and the following results were obtained: TFSWASVTSP, HPDSHQLFIGNLPHEVD, VLSNRPIMFR and GPPRGGMVQKPGFVGRGLA. The data bank search showed a 100% correspondence with G3BP (GenBank accession no. U32519). As described by Parker et al.
Activity on DNA/RNA and RNA/RNA partial duplexes

The RNA/DNA and RNA/RNA substrates were obtained as indicated in Materials and Methods and tested in the same conditions as the DNA/DNA substrates. The results showed that HDH VIII can in fact unwind both RNA/DNA and RNA/RNA substrates with a comparable efficiency. HDH VIII (0.5 U) incubated with 1 ng of DNA/DNA, DNA/RNA or RNA/RNA substrates unwound 12, 7 and 10% respectively of the annealed oligonucleotide (Fig. 6). In the same figure also the activity of 1 U of HDH IV/nucleolin is shown for comparison.

DISCUSSION

The isolation, characterisation and sequencing of HDH VIII show that this is a protein with DNA and RNA helicase activity corresponding to the so-called G3BP, namely a 68 kDa protein encoded by a 3.3 kb mRNA, which had been previously isolated by Parker et al. (14) as an element of the Ras signal transduction pathway; G3BP is in fact a protein binding specifically to the SH3 (Src homology 3) domain of the Ras-GTPase activating protein (GAP) (15,16).

At present, according to the mere amino acid sequence, 31 putative DNA helicases containing the canonical DEAD(H) box have been identified in human cells (17). On the other hand, the functional similarity in this group of proteins cannot be assumed purely on the basis of sequence homology and a direct biochemical demonstration of helicase activity remains essential. In fact, in the case of the HDH VIII sequence we do not observe the typical features of the helicases; we can observe instead the presence of an RNP2 and an RNP3 motif that are typical of the proteins belonging to the hnRNP superfamily (18), as well as of a 23 residue stretch of RGG boxes with a 75% identity with the gly-rich C-terminal of nucleolin, as follows (the letters in bold indicate the homology):
It has to be recalled, in this context, that in our laboratory we have shown that nucleolin is in fact a DNA and RNA helicase (HDH IV) and that its gly-rich C-terminal domain [previously shown to be endowed with RNA-helix-destabilizing properties (19, 20)] is indeed the site of its ATP-dependent unwinding activity. Moreover, also the p68 RNA helicase (21, 22), a DEAD box protein, contains an RGG-rich motif. Interestingly, this domain is often situated at the C-terminal of the protein, possibly a reflection of its functional role.

Several proteins endowed with both DNA and RNA helicase activity are described in the literature, although certainly the RNA unwinding ability is not a universal property of DNA helicases, and vice versa; for example the SV40 T antigen (23), HDH I (13) and HDH IV (10) are all DNA and RNA helicases but several other human DNA helicases, like HDH II (8), HDH VI (24) or HDH VII (Ochem et al., submitted) are devoid of RNA unwinding capacity, whereas in the case of the p68 RNA helicase the DNA unwinding activity is controversial.

HDH VIII is able to unwind with the same, albeit low, efficiency, in a strictly ATP-dependent fashion. DNA, DNA/RNA or RNA partial duplexes in which the annealed oligonucleotide need not have unannealed tails; the presence of an unannealed 3′ stretch approximately doubles the efficiency of unwinding. Several helicases, whether involved in replicative, repair or recombinitive processes, show an analogous preference for fork-like structures. The enzyme is not stimulated by RPA (data not shown) and moves in the 5′ to 3′ direction. The unwinding reaction catalyzed by HDH VIII is observable in vitro only with short (17 bp) duplex DNA molecules whereas, as the length of the duplex DNA increases, the unwinding capacity decreases dramatically; the unwinding of partial DNA duplexes in which the annealed portion is 40 bp long is almost undetectable suggesting that, in vitro, HDH VIII is a non-processive enzyme.

This result is not surprising in view of the possible role of HDH VIII in DNA and RNA metabolism. In fact, the identification of this molecule with G3BP implies that: (i) it is involved in the Ras signal transduction pathway, since it binds specifically (probably with its di-proline motif) to the SH3 motif of the Ras-specific GTPase-activating protein (GAP), a molecule playing an essential role in regulating the level of the proliferation-proficient form of Ras; (ii) its presence in GAP-bound form is strictly proliferation correlated; (iii) it presents, as mentioned above, strong similarity with the family of RNA binding and destabilizing proteins; (iv) it is mainly present in the cytoplasm, but its presence in the nucleus is also significant, since we have isolated it from a nuclear extract. Hence, in view of its RNA helicase activity, it can be speculatively envisaged as playing a function connecting RNAs to upstream signalling molecules like GAP. Since the SH3 motif of GAP appears to be essential for the Ras signal transduction, HDH VIII/G3BP could be a good candidate for linking some aspect of RNA metabolism to cell proliferation. Several hnRNP proteins (25) have been shown to shuttle between nucleus and cytoplasm (26) and these shuttling RNA-binding proteins have been proposed to act as carriers of mRNA out of the nucleus, particularly in the so-called Ran pathway (27) that presents several similarities with the Ras one. Furthermore, the serine-phosphorylated form of G3BP has been reported recently (28) to be able to bind specifically to the 3′-untranslated region of c-myc RNA, and to cleave it, whereas such capacity is nearly abolished in the non-phosphorylated form; the unwinding capacity reported here could conceivably represent a corollary of (or a prerequisite for) the initiation of such specific mRNA turnover. Alternatively, or additionally, one can speculate that the helicase activity could be useful in destabilizing and presenting the RNA to ribosomes and that the presence or absence of bound RNA could modulate the effect of G3BP/HDH VIII on GAP and thus on the activation of Ras.

Conversely, it is not possible to establish at present whether the ability of HDH VIII to also unwind DNA has any functional significance or is merely a quirk of the structure of its active site. Further study on this protein will certainly be necessary to assign it a function in nucleic acid metabolism.

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REFERENCES