Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death

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SUMMARY

CENP-C, one of the few known intrinsic proteins of the human centromere, is thought to play structural as well as regulatory roles crucial to proper chromosome segregation and mitotic progression. To further define the functions of CENP-C throughout the cell cycle we have used the yeast interaction trap to identify proteins with which it interacts. One specific CENP-C interactor, which we have named HDaxx, was characterized in detail and found to be homologous to murine Daxx, a protein identified through its ability to bind the death domain of Fas (CD95). The interaction between CENP-C and HDaxx is mediated by the amino-terminal 315 amino acids of CENP-C and the carboxyl-terminal 104 amino acids of HDaxx. This region of Daxx is responsible for binding to death domains of several apoptosis signalling proteins. The biological significance of the interaction between CENP-C and HDaxx was confirmed by immunofluorescence colocalization of these two proteins at discrete spots in the nuclei of some interphase HeLa cells. We discuss the functional implications of the interphase-restricted association of HDaxx with centromeres.

Key words: Interphase centromere, CENP-C, HDaxx

INTRODUCTION

The centromere is the chromosomal region responsible for the precise and accurate segregation of eukaryotic genetic material during mitosis and meiosis (reviewed by Pluta et al., 1995). Centromeres direct the segregation of mitotic chromosomes through a differentiated multilayered structure called the kinetochore, which serves as the binding site for spindle microtubules at prometaphase and for the mechanochemical motors that move chromosomes along those microtubules during anaphase. It is becoming increasingly clear that kinetochores also act as cellular sensors that monitor proper requirements necessary for progression through the cell cycle. As the last region of replicated chromosomes to remain paired prior to disjunction, centromeres both monitor the bipolar attachment of chromosomes to the spindle and regulate the separation of the sister chromatids at the metaphase-anaphase transition. Centromeres defective in any of these mitotic functions can result in aneuploidy, a condition that accounts for a high percentage of early embryonic lethality in humans.

A complete understanding of how the centromere coordinates these roles requires comprehensive knowledge of its biochemical associations at all stages of the cell cycle, yet the list of known human centromere proteins (CENPs) is brief. Many CENPs associate transiently with centromeres only during mitosis, and as the cell exits mitosis and enters interphase these proteins are degraded or transferred from the kinetochore to the mitotic spindle (Brinkley et al., 1992; Allshire, 1997). Some, such as cytoplasmic dynein and kinesin-related CENP-E, supply the microtubule-binding and motor functions carried out by the kinetochore (Wordeman et al., 1991; Wood et al., 1997; Schaar et al., 1997). Others, such as the 3F3/2 antigens and the spindle assembly checkpoint proteins MAD2 and BUB1 are involved in tension sensing and cell cycle signaling (Campbell and Gorbsky, 1995; Li and Benezra, 1996; Taylor and McKeon, 1997). Less is known about the functions of others, including the INCENP chromosomal passengers (Earnshaw and Cooke, 1991), kinesin-related MCAK (Wordeman and Mitchison, 1995) and CENP-F/mitosin (Casiano et al., 1993; Rattner et al., 1993; Zhu et al., 1995).

Despite the absence of the mitotic-specific CENPs, condensed centromeres persist as discrete chromatin domains throughout the cell cycle (Moroi et al., 1981; Matsumoto et al., 1989b; Cooke et al., 1990). Nevertheless the interphase centromere, unlike its mitotic counterpart, is functionally ill-
defined partly because so few of the proteins that comprise it are known. CENPs-A, -B and -C, initially identified as autoantigens in patients with scleroderma spectrum disease (Moroi et al., 1980; Brenner et al., 1981), are the only human proteins known to remain associated with centromeric DNA at all stages of the cell cycle ( Cooke et al., 1990; Sullivan et al., 1994; Knehr et al., 1996). While the relationship between this immunoreactivity and the etiology of systemic sclerosis is unclear, human autoimmune sera containing anti-centromere antibodies (ACA) were used to clone and characterize these intrinsic CENPs. CENP-A, a novel centromere-specific core histone related to histone H3, was recently immunolocalized to the inner kinetochore plate ( Sullivan et al., 1994; Warburton et al., 1997). CENP-B is a sequence-specific α-satellite DNA binding protein localized throughout the centromeric heterochromatin located beneath the kinetochore (Cooke et al., 1990; Earnshaw et al., 1987; Matsumoto et al., 1989a). Its functional significance for centromeres remains unclear since it is undetectable on human Y chromosomes (Earnshaw et al., 1991; Matsumoto et al., 1993) and it was recently reported that the mouse CENP-B gene is not essential for viability ( Kapoor et al., 1997). CENP-C is a structural component of the inner kinetochore plate ( Saitoh et al., 1992). All the intrinsic human CENPs display homology with yeast proteins involved in centromere biology, indicating that their functions are important and likely to be conserved between species (Brown, 1995; Meluh and Koshland, 1995; Stoler et al., 1995; Lee et al., 1997; Halverson et al., 1997). However, immunofluorescence studies of human dicentric chromosomes, which are mitotically stable because they are functionally monocentric, have revealed that CENPs-A and -C are found only at active centromeres, while CENP-B is detectable at both the active and inactive centromeres (Earnshaw et al., 1989; Page et al., 1995; Sullivan and Schwartz, 1995). The fact that CENPs-A and -C are present throughout the cell cycle, but their presence specifically correlates with mitotic centromere activity indicates that they may participate in the very earliest signaling and/or marking events in interphase that eventually determine mitotic centromere assembly and function. Yet it remains unclear whether the interphase centromere relies on these proteins merely to mark the chromosomal region destined to assemble the kinetochore at mitosis or whether they have other unsuspected functions specific to interphase.

In order to probe centromere function throughout the cell cycle, we have used the yeast interaction trap to search for proteins that interact with CENP-C. We have identified and characterized an unanticipated interaction of CENP-C with HDaxx, a human protein whose mouse homolog has been proposed to modulate Fas-mediated apoptosis. Our finding that HDaxx colocalizes with interphase centromeres suggests that centromeres may play a role in regulating cellular responses to apoptotic stimuli.

**MATERIALS AND METHODS**

**DNA constructs used in the yeast interaction trap**

pTCA TG (Pluta and Earnshaw, 1996) was digested with NdeI, treated with T4 DNA polymerase to produce blunt ends, then digested with BamHI, and the resulting insert containing the full-length CENP-C open reading frame was cloned into EcoRI-digested, T4 DNA polymerase-treated pEG202 (Gyuris et al., 1993), which was also digested with BamHI. The correct reading frame of the resulting construct, LEXA:CENP-C1-943 (in which the numbers refer to the amino acid residues of CENP-C), was confirmed by DNA sequence analysis. The -945 bp fragment produced by digesting pTCA TG with Ndel, followed by treatment with T4 DNA polymerase and then BglII, was cloned into the same vector as above to create LEXA:CENP-C1-315. The carboxyl-terminal third of the CENP-C ORF was PCR-amplified from pTCA TG using Vent DNA Polymerase (New England Biolabs) and oligonucleotide primers CENPC14 (5′-GG-GAATTCAGATCTACAAGAGCCTC-3′) and CENPC2831-2816 (5′-GGGAAATTCATTTTTATCTGAG-3′). The resulting fragment was gel-purified, digested with EcoRI (sites underlined) and cloned into the EcoRI site of pEG202 to create LEXA:CENP-C635-943. Oligonucleotide primers CENPC12 (5′-GGGAAATTCATGGGATCCTGGATCCTTGATTAC-3′) and CENPC15 (5′-CATTCTTCCAGTTCCATTCTTTGAGCTTC-3′) were used to PCR-amplify the middle third of the CENP-C ORF from pTCA TG. The resulting fragment was EcoRI- and Xhol-digested, gel-purified and cloned into EcoRI- and Xhol-digested pEG202 to create LEXA:CENP-C316-643. DNA constructs expressing LEXA:CENP-C180-468 and LEXA:CENP-C462-802 were provided by J. Tomkiel (Wayne State University).

To express HDaxx in yeast as an activation domain-tagged protein, the full HDaxx ORF was PCR-amplified from pBS/D31-1 using oligonucleotide primers CB22P (5′-GCGGAATTCCTATGCGCACCCTACAAC-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′), and the resulting 2.4 kb fragment was digested with EcoRI and SalI (which cuts in the library adaptor sequence at the 3′ end of the HDaxx ORF), gel-purified and cloned into EcoRI- and Xhol-digested pJG4-5 (Gyuris et al., 1993) to produce pJG4-5/HDaxx1-740.

**Yeast interaction trap screen and quantitative β-galactosidase assays**

The yeast interaction trap screen was performed essentially as described ( Golemis et al., 1996). ~20 μg of a poly(dT)-primed HeLa cell cDNA library in pJG4-5 (Gyuris et al., 1993) was transformed into yeast strain EGY48 which contained LEXA:CENP-C1-943 and pSH18-34, yielding ~5×10⁶ primary transformants which were pooled, washed in sterile dH₂O, resuspended in sterile 65% (v/v) glycerol, 0.1 M MgSO₄, 25 mM Tris-HCl, pH 7.4, and frozen in small aliquots at ~70°C. ~3×10⁷ cfu containing LEXA:CENP-C1-943, pSH18-34 and library plasmids were plated on Gal/Raf/CM-ura-his-trp-leaf plates to screen for transcriptional activation of the 3LexAop:Leu2 gene engineered into the yeast strain. 156 Leu+ yeast colonies were picked to a Glu/CM-ura-his-trp master plate, then replica-plated to Glu/CM-ura-his-trp-leaf, Glu/Xgal/CM-ura-his-trp, and Gal/Raf/Xgal/CM-ura-his-trp plates to test for galactose-dependent leu2 and lacZ expression. 80 Leu+ colonies displayed the interaction phenotype when plated on these media (growth on Gal/Raf-ura-his-trp-leaf but not on Glu-ura-his-trp-leaf, and blue colony color on Gal/Raf-Xgal, but white on Glu/Xgal); of those, 32 were randomly selected for further study and 23 were subsequently recovered as library plasmids in Escherichia coli strain JBe15. Specificity of the interaction between the CENP-C bait and the individual library plasmids was verified by transforming each isolated library plasmid into yeast strain EGY48 containing reporter plasmid pSH18-34 and either LEXA:CENP-C1-943 or pRFH1M1 (Golemis and Brent, 1992; Zervos et al., 1993), and testing for colony color on Gal/Raf/Xgal/CM-ura-his-trp-leaf plates and growth on Gal/Raf/CM-ura-his-trp-leaf plates. Library plasmids giving the phenotype expected for specific interaction with the CENP-C bait were used to transform E. coli strain DH5α, from which they were isolated for further study. β-Galactosidase activity of yeast transformants (3–5 isolates of each strain) grown in liquid culture was determined using ONPG (o-nitrophenyl β-D-galactopyranoside) as substrate (Reynolds and Lundblad, 1989).
HeLa cDNA library screen and northern blot analysis
The ~1 kb insert from library plasmid pJG4-5/CBP1 was PCR-amplified using oligonucleotide primers BCO2 (5'-GAGAAGCGCAACCTTGGATGGAG-3') and 5'ATGJG4-5'TAGTTGCTGATGAGAATTCC-3', gel-purified and 3'P-labeled by random priming (Feinberg and Vogelstein, 1983) and used as probes in cDNA library screening and northern blot analysis. cDNA clones encoding the HDaxx message were isolated by screening ~1.6x10^5 pfu from an oligo(dT) + random-primed HeLa 5'-Stretch Plus cDNA library (Clontech). Plaque lifts and hybridizations were performed according to the manufacturer’s instructions. cDNA inserts were excised with EcoRII from phage DNA prepared from plaque-purified positives and subcloned into the EcoRI site of Bluescript in both orientations to produce pBS/D31-1 and pBS/D31-2. DNA sequence analysis of this insert revealed that the bona fide 5' end of the phage cDNA insert had not been recovered as part of this EcoRI fragment, and it was subsequently recovered by subcloning the ~800 bp fragment resulting from digestion of D31 phage DNA with NotI (which cuts in the library adaptor sequence) and PsrI into NotI- and PsrI-digested Bluescript.

Total RNA was isolated from HeLa (S3) cells grown in spinner culture (Chomczynski, 1996). Electrophoresed on a 1% formaldehyde-agarose gel, transferred in 20× SSC to a nitrocellulose filter and hybridized as above. All filters were washed once for 5 minutes at 25°C in 2x SSC, 0.1% SDS, then twice for 5 minutes at 25°C in 0.2x SSC, 0.1% SDS, and twice for 15 minutes at 50°C in 0.1x SSC, 0.1% SDS.

DNA sequence determination and analysis
Double-stranded DNA was sequenced manually on both strands using Sequenase Version 2.0 (US Biochemicals) and SequiTherm EXCEL (Epicentre Technologies) DNA Sequencing kits and [α-35S]dATP. DNA and protein sequences were analyzed using MacVector 6.0 and AssemblyLIGN 1.0.5. Homology searches of the NCBI non-redundant protein and nucleotide databases were performed using the BLAST program (Altschul et al., 1990).

Bacterial fusion protein expression and antibody production
Inserts from several members of the largest group of library plasmids recovered in the two-hybrid screen were PCR-amplified using oligonucleotide primers BCO2 and 5'ATGJG4-5 phosphorylated with T4 polynucleotide kinase and cloned into the NotI site of pet16b (Novagen) which was treated with T4 DNA polymerase and calf intestine alkaline phosphatase (Boehringer Mannheim). Correct orientation of inserts with respect to the T7 promoter was confirmed by PCR amplification using oligonucleotide primers T7 and BCO2. Histidine-tagged fusion protein expression was induced in E.coli strain BL21(DE3) at 37°C for 2 hours with 1 mM IPTG, after which the cells were collected, boiled in SDS, then sonicated to produce a lysate from which fusion proteins were affinity-purified on His-Bind resin (Novagen) according to the manufacturer’s instructions. 1 mg of each purified protein was dialyzed against 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2% SDS, then precipitated on ice with 20% TCA. Following centrifugation, the pellets were washed with 90% acetone, 10% 0.1 N HCl and air-dried. Pellets were resuspended in PBS, pooled and used as immunogen by Covance Research Products (Denver, PA) to generate a rabbit polyclonal antibody against amino acids 510-740 of HDaxx (anti-HDaxx).

Cell culture, subcellular fractionation and western blotting
Suspension cultures of HeLa (S3) were grown in RPMI 1640 with 5% fetal calf serum; adherent HeLa (JW) and U2OS (human osteosarcoma) cells were grown in monolayer cultures on glass coverslips in DMEM with 10% fetal bovine serum. Subcellular fractionation and immunoblotting were carried out as described by Earnshaw and Rattner (1991) with the following modifications.

RESULTS
Isolation of HDaxx
The yeast interaction trap assay was used to screen a galactose-inducible HeLa cDNA expression library for proteins that interact with CENP-C (Gyuris et al., 1993). Primary yeast transformants containing LEXA:CENP-C1-943, lacZ reporter plasmid pSH18-34 and HeLa cDNA library plasmids were plated on selective medium for galactose-dependent transcriptional activation of the LexAop-LEU2 reporter gene engineered into the genome of yeast strain EGY48. 156 of the resulting Leu+ yeast colonies were tested for their ability to induce galactose-dependent lacZ expression from the LexAop-lacZ reporter gene contained on pSH18-34 in this strain. Approximately 50% of the Leu+ transformants displayed the expected phenotype for interaction when plated on selection medium (see Materials and Methods). Of those, 32 transformants were randomly selected for further study and 23 were subsequently recovered as plasmids in E.coli. 18 of the isolated library plasmids reproduced the specific interaction phenotype when retransformed into yeast co-expressing the original CENP-C bait, but not in yeast co-expressing an irrelevant bait, LEXA:Drosophila bicoid, thus fitting the initial criteria for CENP-C interactors.

The 18 putative CENP-C interactors were categorized into

Partially purified HeLa chromosomes were prepared from a HeLa culture that had been blocked in mitosis with 6 nM vinblastine (Sigma). HeLa nuclei were prepared using an unblocked log phase culture; the pellet resulting from the first spin following Dounce lysis consisted of a crude nuclear fraction, while the supernatant contained the crude cytoplasmic fraction. Immunodetection on western blots was carried out using either 125I-labeled Protein A or horseradish peroxidase-conjugated anti-rabbit IgG and ECL (Amersham Corp.).

GFP fusion constructs, transfections and immunofluorescence
The first 573 codons of HDaxx were PCR-amplified from pBS/D31-1 using primers CCBPBNA TG and CCBP11 (5'-ACAGAGGAGGGGTATC-3'), digested with BglII and HindIII, and cloned into BglII- and HindIII-digested pEGFP-C1 (Clontech) to generate pGFP:HDaxx1-573. pGFP:HDaxx1-740 was constructed by replacing the small HindIII-EcoRI fragment of pGFP:HDaxx1-573 with the 700 bp HindIII-EcoRI fragment of pBS/D31-1. The 680 bp HindIII-BamHI fragment from pBS/D31-1 was subcloned into HindIII- and BamHI-digested pEGFP-C1 to make pGFP:HDaxx573-740.

GFP constructs were introduced into HeLa or U2OS cells by lipid-mediated transfection using Lipofectin reagent (Bethesda Research Labs; Pluta et al., 1992) or by electroporation (Mackay et al., 1993). Indirect immunofluorescence detection of HDaxx and human centromere proteins was performed on cells grown on coverslips and then fixed in 3% paraformaldehyde for 5 minutes as previously described (Pluta et al., 1992; Earnshaw and Rattner, 1991). CENP-C was detected with polyclonal rabbit anti-HDaxx (Tomkiel et al., 1994) followed by biotinylated goat anti-rabbit IgG followed by Texas Red conjugated to streptavidin; human centromeres were detected using human autoimmune serum GS, which recognizes CENPs -A, -B and -C (Earnshaw and Rothfield, 1985), followed by FITC-conjugated anti-human IgG; DNA was stained with 4',6-diamidino-2-phenyl-indole (DAPI). Slides were examined on a Leica DM IRB microscope and images were collected using a Photometric Sen Sys camera driven by IP Labs image processing program.

CENP-C binding protein, HDaxx 2031
northern blot of total HeLa RNA hybridized with a 32P-labeled cDNA insert from CBP1, of the human HDaxx cDNA. (A) A single size message of ~2.4 kilobases was detected on a Northern blot analysis of HDaxx mRNA, and DNA and deduced amino acid sequence localization signals (Nakai and Kanehisa, 1992) are indicated by double underlines. Two regions with high probability of forming coiled coils (Lupas, 1996) are corresponding to the cDNA insert from CBP1. Conceptual translation of the major open probe was used to isolate phage D31 from a Agt11 HeLa cDNA library. The entire 2.477 bp DNA sequence of the D31 insert is shown. The dashed underline indicates the DNA sequence corresponding to the cDNA insert from CBP1. Conceptual translation of the major open reading frame, corresponding to the 740 residue HDaxx protein, is shown below the DNA sequence. Two regions with high probability of forming coiled coils (Lupas, 1996) are indicated by a single underline; two short basic motifs predicted to direct nuclear localization signals (Nakai and Kanehisa, 1992) are indicated by double underlines.
groups based on a comparison of their DNA sequences. The largest group consisted of 11 non-identical library plasmids that were related to each other by DNA sequence. Because database searches revealed that the gene encoded by this group of library plasmids was novel, the full-length cDNA encoding this interactor was isolated for further study. The cDNA insert of one member of this group of library plasmids, CBP1, was used as a probe for northern blot analysis of total HeLa RNA and it detected a single message of ~2.4 kb (Fig. 1A). The same probe was used to screen a λgt11 HeLa cDNA library, and among the cross-hybridizing phage recovered was one, named D31, that contained an insert of ~2.5 kb. DNA sequence analysis of this insert revealed a single long open reading frame capable of encoding a protein of 740 amino acids with a calculated molecular mass of 81.3 kDa, which we initially named CCBP (for CENP-C binding protein; Fig. 1B).

Conceptual translation of the CCBP open reading frame predicts a protein that is highly hydrophilic and acidic (calculated pI = 4.6), and contains two regions with high concentrations of glutamic and aspartic acid residues (Fig. 2). The minor acidic region, near the amino terminus, is 7 amino acids in length (100% glu + asp), while the major acidic region in the middle third of the protein is 52 amino acids in length (78% glu + asp). Computer analyses of the CCBP amino acid sequence also revealed two short basic motifs predicted to represent nuclear localization sequences. The shaded region represents the domain of HDaxx sufficient for interaction with CENP-C: this corresponds to the smallest cDNA insert that was recovered in the yeast interaction trap assay. The region of HDaxx used to produce the polyclonal antibody (amino acids 510-740) is indicated as anti-HDaxx. The entire region of HDaxx designated by the partially dashed line above the HDaxx open reading frame (amino acids 493-740) was recovered in a yeast two-hybrid screen for mouse Fas death domain interactors, while the solid part of that line indicates the amino acids (625-740) sufficient for interaction with Fas (Yang et al., 1997). (C) The predicted structure of GFP:HDaxx fusion proteins expressed in vivo. The name of each construct indicates, in numbers, the amino acid residues of HDaxx expressed in the fusion protein.

The amino terminus of CENP-C interacts with HDaxx

We also used the yeast interaction trap assay to determine the region of CENP-C responsible for its interaction with HDaxx. Various baits, consisting of full-length CENP-C and five overlapping domains constituting the entire CENP-C open reading frame, were assayed for their interaction with HDaxx by monitoring galactose-inducible activation of the lacZ reporter plasmid pSH18-34 in yeast (Fig. 4). Blue colony color on Xgal-containing galactose plates was initially used as a gross indicator of interactions between HDaxx and CENP-C domains. These interactions were verified and their relative strengths quantified by measuring β-galactosidase activity of liquid yeast cultures (Fig. 4A). By this criterion, full-length HDaxx interacted with full-length CENP-C as well as with the two amino-terminal proximal CENP-C domain constructs encompassing amino acids 1-315 and 180-468.

During testing of the various LEXA:CENP-C baits, we noted that some had intrinsic transcription activation activity,
resulting in low to mid-level expression of the lacZ reporter gene under non-inducing conditions (glucose) as well as in the absence of specific activation-tagged prey. In order to determine to what extent such self-activation could account for the interaction phenotype observed above, the following experiment was performed. Full-length CENP-C, as well as the two amino-terminal proximal CENP-C domain constructs that also appeared to interact with HDaxx and either activation-tagged HDaxx1-740 or empty vector JG4-5 as prey. Individual transformants were then grown in parallel in liquid culture under conditions that repressed (glucose) or induced (galactose) expression of the prey. Quantitative β-galactosidase assays were then performed to determine the relative increase in lacZ expression when HDaxx was specifically expressed as the prey. Thus, β-galactosidase enzyme units measured for cells grown in glucose (repressing conditions) reflect the intrinsic activation activity of a particular bait, while enzyme units measured for cells grown in galactose (inducing conditions) reflect the activation activity resulting from the interaction of a bait with a specific prey.

Results of this experiment, shown in Fig. 4B, are summarized as follows. First, while LEXA:CENP-C1-943 produced negligible enzyme activity when grown in glucose, LEXA:CENP-C1-315 and LEXA:CENP-C180-468 each showed modest, but detectable enzyme activity when grown in glucose, indicating the ability of these latter two baits to activate transcription of the lacZ reporter by themselves. Second, all three of the baits produced increased enzyme activity when grown in galactose, conditions that induce expression of the prey, which consisted of either the activation domain itself (empty vector) or activation domain-tagged HDaxx. However, the most significant increases occurred in cells co-expressing the CENP-C-derived bait and HDaxx. Specifically, a ~130-fold increase in activity was measured for cells expressing both LEXA:CENP-C1-943 and HDaxx; a 40-fold increase for cells expressing LEXA:CENP-C1-315 and HDaxx; and a 9-fold increase for cells expressing

| HDaxx | 1 MATANSIIVLDDEDEEAAQAQPFSHL2PNAASPG-------AEAPSSSFHGARGSSGGGKCKYKLENKFE | 68 |
| mDaxx | 1 MATDDSIIVLDDDEDEEAAQAQPFSHLPPAPSTPQGPLQQATGSEFPRVGDGSNSGSKCKYKLENKFE | 74 |
| HDaxx | 69 EFLELCMQADTEPHFVFPLHVRQDGSSFLAESFCNISLRVLSRSAVPAKIVYNEICTVLAGHASSK | 142 |
| mDaxx | 75 EFLELCQETDSDHPEVFPLHLQRGFQSSFLAESFCNISLRVLSRSAVPAKIVYNEICTVLAGHASSK | 148 |
| HDaxx | 143 LNLAPAATTSPSNQPHTLPSLTDPTNAASTASQPSRTGRSQRLQLQPLLALVGVAEIRLQGEKEDLSEDSE | 216 |
| mDaxx | 149 LNLAPAATTSPSNQPHTLPSLTDPTNAASTASQPSRTGRSQRLQLQPLLALVGVAEIRLQGEKEDLSEDSE | 222 |
| HDaxx | 217 DPDSAYLQERARLKILRFYGLFRKLRCSSLTGVRIEQIRPBYPETVPVRNLIRLNGPDPITFDYGDVL | 290 |
| mDaxx | 223 DPDSAYLQERARLKLFRYGLFRKLRCSSLTGVRIEQIRPBYPETVPVRNLIRLNGPDPITFDYGDVL | 296 |
| HDaxx | 291 RAVEKAARRHLGSLRPQQLMQADAFRQGRLQERRHLDLILYFCLHLDDRYGPDLSVLPDVLRRRRE | 364 |
| mDaxx | 297 RAVEKAARRHLGSLRPQQLMQADAFRQGRLQERRHLDLILYFCLHLDDRYGPDLSVLPDVLRRRRE | 370 |
| HDaxx | 365 RSLAMSLRDLIESVLQAMDQGKLQSEEGRRKRKVLQRQGTSNADTPAESLDSEQGSPMQQPCSPQAEATDDDE | 438 |
| mDaxx | 371 RSLAMSLRDLIESVLQAMDQGKLQSEEGRRKRKVLQRQGTSNADTPAESLDSEQGSPMQQPCSPQAEATDDDE | 444 |
| HDaxx | 439 DO-------EREDREDEEEEEEEEEEETEEEEDEEEDEEEEEDEEEDEEEDEEEEAAAGKGDKSPMSLQLIE | 503 |
| mDaxx | 445 DDDDDDDDDDDDEE---EEEEEEEEEDEDEEDEEHDELQHEDGQGD---EEE----DDDNEGDSRESPSCDFD | 510 |
| HDaxx | 504 NEKNLEPGKQISRSSEGGQQNKRIVGSPSILPEELAPEPSIDASENPSQPGPEELTHEEESPQSLPSLEIELALPLD | 577 |
| mDaxx | 511 HRNNEPAEGLRTPE---QQRKLTELSTPEPSGGASLDQSDSVEGSPMQQPCSPQAEATDDDE | 582 |
| HDaxx | 578 TPSSVETDISSRQRSEQEPFETTVLLENGAMVSTSFNGVSPHNGDSGPPQKRRKRTTGTSSGPLNSYVER | 651 |
| mDaxx | 583 TPSVSETDISSRQRSEQEPFETTVLLENGAMVSTSFNGVSPHNGDSGPPQKRRKRTTGTSSGPLNSYVER | 658 |
| HDaxx | 652 QRSSVEINGKSKICLTLPSSPSLASLAPAVDSVTRDSVPSHGVEVSSTCLCPSARLSPHTQSQPPRPGCTKTSVA | 724 |
| mDaxx | 652 QRSSVEINGKSKICLTLPSSPSLASLAPAVDSVTRDSVPSHGVEVSSTCLCPSARLSPHTQSQPPRPGCTKTSVA | 729 |

Fig. 3. HDaxx is highly homologous to mouse Daxx. HDaxx and mouse Daxx protein sequences were aligned using identity matrix of the Clustal W(1.4) sequence alignment program (MacVector 6.0) with default settings. Identical amino acids are indicated by ; similar amino acids are indicated by :. Overall identity is 73%. The amino termini of the proteins (first 440 residues) show 83% identity; the regions immediately following the major acidic domains show 54% identity over an aligned length of 265 residues. Underlined amino acids are sufficient for interaction with CENP-C. The same region plus the 7 additional residues indicated by a dotted underline are sufficient for interaction with Fas (Yang et al., 1997).

| | 700 |
| | 740 |
| | 739 |
Fig. 4. HDaxx interacts with the amino-terminal 315 amino acids of CENP-C. (A) Five different CENP-C-derived baits and two control baits were tested for their ability to interact with full-length HDaxx in the yeast two-hybrid assay. Interaction was monitored by colony color on X-gal-containing galactose plates and was quantified by measuring β-galactosidase units for strains grown in liquid cultures. The domains of CENP-C that were tested are indicated schematically. Numbers refer to the amino acid residues of CENP-C expressed as LexA fusion proteins; relative positions of putative nuclear localization sequences in the CENP-C open reading frame are marked by asterisks; the hatched box indicates the region of CENP-C responsible for targeting the protein to centromeres in vivo (Yang et al., 1996). Blue colony color on X-gal containing galactose plates indicates an interaction between the bait and prey proteins, while white colony color indicates the absence of an interaction. The relative strength of protein interactions was determined by measuring β-galactosidase enzyme units resulting from expression of the lacZ reporter gene. (B) EGY48 containing the lacZ reporter plasmid was cotransformed with the indicated combinations of CENP-C-derived baits and activation-tagged prey vector, JG4-5, containing or lacking full-length HDaxx. β-Galactosidase units were measured and averaged for 3-5 independent transformants, each of which was tested under conditions that repressed (glucose) and induced (galactose) expression of the activation-tagged prey. Fold increase is the ratio of β-galactosidase enzyme units determined for cells containing a particular bait and prey in grown in galactose divided by that determined for the same cells grown in glucose, and indicates the relative strength of interaction independent of intrinsic activation activity of the bait.

LEXA: CENP-C 180-468 and HDaxx. In contrast, a constant 2-fold increase in enzyme activity was measured for cells co-expressing any of the CENP-C-derived baits and the empty prey vector JG4-5 when grown in inducing conditions. Thus, despite this low intrinsic transcriptional activation activity of the two amino-terminal proximal CENP-C baits, the most significant increase in activation was observed when the first 315 amino acids of CENP-C was co-expressed with HDaxx, indicating that this region is responsible for the interaction of CENP-C with HDaxx.

The HDaxx-interacting region of CENP-C does not overlap with any of the characterized functional domains of CENP-C, which include a centrally-located region responsible for targeting the protein to centromeres, an overlapping domain that has non-specific DNA-binding activity in vitro, and a carboxyl-terminal domain with in vitro dimerization activity (Yang et al., 1996; Sugimoto et al., 1997). A phosphorylation consensus site for p34^cdk2, SPSK located at residues 73-76, is the only distinguishing feature of amino terminus of CENP-C (Saitoh et al., 1992), but phosphorylation of this site during the cell cycle has not been studied, and its functional significance is not known. It has been reported that the amino terminus of CENP-C possesses oligomerization activity in vitro (Sugimoto et al., 1997) as well as a novel instability activity that prevents toxic accumulation of high levels of CENP-C when ectopically expressed in baby hamster kidney cells (Lanini and McKeon, 1995). However, a similar instability activity has not been observed for CENP-C when it is overexpressed in human cells (Yang et al., 1996).

**HDaxx is a predominantly nuclear protein and is absent from isolated mitotic chromosomes**

To begin to address the biological significance of the interaction detected in yeast between HDaxx and CENP-C, we determined the subcellular distribution of HDaxx in human tissue culture cells. A log phase suspension culture of HeLa cells was mechanically separated into nuclear and cytoplasmic fractions by differential centrifugation of hypotonically swollen, Dounce-homogenized cells. An independent culture of HeLa cells was blocked in mitosis with vincristine, and similarly treated to produce partially purified mitotic chromosomes. Aliquots of each fraction (nuclei, cytoplasm and chromosomes) were immunoblotted with a rabbit polyclonal antibody produced against the carboxyl-terminal 230 amino acids of HDaxx (anti-HDaxx; see Materials and Methods). To monitor the efficiency of the subcellular fractionation, CENP-C was detected in a parallel blot of identical fractions with a specific polyclonal antibody (Tomkiel et al., 1994).

HDaxx migrated in SDS polyacrylamide gels with a relative molecular mass of ~116 kDa and fractionated almost quantitatively with nuclear proteins (Fig. 5). The minor HDaxx signal seen in the lane containing the cytoplasmic protein fraction is probably due to contamination by nuclei damaged during purification, as a small amount of CENP-C was also detected in this fraction. Nuclear fractionation of HDaxx was unexpected since its putative homolog, mouse Daxx, interacts with the cytoplasmic death domain of Fas (CD95), a plasma membrane-bound cell surface receptor. However, this result is consistent with the presence of two putative nuclear localization signals within the HDaxx amino acid sequence (Fig. 1B), HDaxx was not detectable to an appreciable extent in fractions prepared from partially purified mitotic chromosomes, suggesting either that it is not a chromosomal protein during mitosis, that it is easily dissociated from condensed mitotic chromosomes during the biochemical

**Table A. LexA:CENP-C Baits + HDaxx1-740:**

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**Control Baits + HDaxx1-740:**

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**Table B. LexA:CENP-C Bait + Prey:**

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fractionation process, or that its association with chromosomes is disrupted by treatment of cells with vinblastine. In contrast, CENP-C fractionated as expected with nuclei and with partially purified chromosomes.

The aberrant migration of endogenous HDaxx in SDS polyacrylamide gels is most likely due to the anomalous electrophoretic behavior of the clustered charges present in the acidic domains of the protein, a property that has been noted for other acidic clusters, such as those found in CENP-B (Earnshaw, 1987; Earnshaw et al., 1987). The size of endogenous HDaxx observed by western blotting is consistent with that obtained by in vitro translation of the HDaxx open reading frame and is similar to that observed for in vitro-translated mouse Daxx (AFP, data not shown; Yang et al., 1997). The smaller molecular mass species detected with this antibody probably represent proteolytic breakdown products of the full-length protein generated during preparation of the samples rather than immunologically related proteins present in cells, as bands of similar size were observed in long exposures of $^{35}S$-labeled HDaxx produced by in vitro translation (data not shown).

**HDaxx colocalizes with a subset of interphase centromeres in human nuclei**

To further investigate the intracellular distribution of HDaxx, indirect immunofluorescence was performed using anti-HDaxx to detect the endogenous protein in fixed HeLa cells. An example of the variety of HDaxx staining patterns observed in interphase cells is shown in Fig. 6. As predicted by our biochemical fractionation experiments, HDaxx was detected exclusively in nuclei, where it gave a diffuse nuclear staining pattern that was usually excluded from nucleoli. Many cells also displayed a heterogeneous punctate pattern superimposed on this overall pattern of diffuse nuclear staining that was never observed for cells stained with the corresponding rabbit pre-immune serum (data not shown). The same nuclear staining patterns were observed by fluorescence microscopy of live HeLa cells transfected with GFP:HDaxx1-740, a construct expressing the full-length HDaxx open reading frame fused to GFP, indicating that neither the nuclear localization nor the punctate staining observed with anti-HDaxx were artifacts of the procedures used to fix or permeabilize cells for indirect immunofluorescence (Figs 7K, 9B).

The punctate staining pattern we observed in a number of interphase cells stained with anti-HDaxx was highly reminiscent of that produced by human autoimmune serum containing anti-centromere antibodies (ACA) that recognize the three intrinsic human centromere proteins, CENPs-A, -B and -C (Earnshaw and Rothfield, 1985). We therefore used anti-HDaxx and human ACA to perform double immunofluorescence labeling in order to simultaneously localize HDaxx and centromeres in human tissue culture cells. In general, anti-HDaxx detected fewer nuclear spots in either HeLa or U2OS cells than did ACA (Fig. 7A-D and E-H, respectively). In addition, we noted that the HDaxx spots often appeared less discrete than those produced by human ACA (see for example, Fig. 7F). However, when the individual images produced by staining cells with anti-HDaxx and ACA were merged, it was clear that many, if not most, of the spots obtained with the two anti-sera were superimposable indicating that HDaxx colocalizes with centromeres in interphase nuclei (Figs 7D,H, 8G-I). Interestingly, the number of spots that colocalized varied from cell to cell. In some cells, many HDaxx spots were often juxtaposed in very close proximity to centromeres, but could not be superimposed with them. We obtained similar results in fixed cells expressing transfected GFP:HDaxx1-740 in which HDaxx was detected by GFP fluorescence and endogenous CENP-C was specifically detected by indirect immunofluorescence using anti-CENP-C (Fig. 7I-L). Thus, the intimate juxtaposition of HDaxx and CENP-C in human cells validates the interaction we detected between these two proteins in yeast.

Results from our biochemical fractionation of HeLa cells also suggested that HDaxx might not be a component of condensed mitotic chromosomes (Fig. 5). This was confirmed by indirect immunofluorescence localization of HDaxx in fixed mitotic HeLa cells. Cells in prophase (Fig. 8A-C) and metaphase (Fig.
8D-F) displayed diffuse overall staining but none of the punctate pattern seen in interphase cells and likewise showed no evidence of HDaxx colocalization with centromeres. This was also the case for cells in anaphase and telophase (data not shown). In contrast, the punctate pattern of HDaxx staining and centromere colocalization was apparent in cells that had exited mitosis and were inferred to be in G1 (by the presence of a residual midbody, decondensed DNA and dispersal of unduplicated centromeres) or early S phase (Fig. 8G-I).

The absence of punctate HDaxx staining in some interphase nuclei and in mitotic cells prompted us to investigate whether levels of this protein fluctuate during the cell cycle. Equivalent amounts of protein lysates prepared from HeLa cells that had been synchronized in the cell cycle by mitotic shake-off were immunoblotted with anti-HDaxx (Fig. 8J; Monteiro and Mical, 1996). The steady-state level of HDaxx did not vary appreciably as cells progressed from mitosis through G1, S and G2 of the cell cycle, and into the next mitosis. Thus we conclude that the absence of HDaxx spots in some nuclei (as well as the presence of spots in others) may be controlled by cell cycle-specific post-translational modifications of HDaxx (and/or proteins with which it interacts), rather than by its specific degradation.

Independent domains of HDaxx form nuclear spots in vivo

To begin a functional dissection of HDaxx, the full-length open reading frame and amino- and carboxyl-terminal deletions were fused to green fluorescent protein (GFP) of A. victoria (Fig. 2C) and their expression in live HeLa cells following transfection was monitored by fluorescence microscopy. Cells transfected with the empty GFP vector displayed fluorescence throughout the entire cell, with no specific accumulation in nuclei and never in nuclear spots (Fig. 9A). In contrast, a diffuse fluorescent signal accumulated exclusively in the nuclei of cells transfected with GFP:HDaxx1-740 and GFP:HDaxx573-740 (Fig. 9B and E, respectively) and many cells expressing those constructs also displayed nuclear spots. This pattern was the same as that which we observed for endogenous HDaxx detected in fixed cells by indirect immunofluorescence (Fig. 7A-H). The nuclear spots produced by expression of GFP:HDaxx573-740 colocalized with interphase centromeres stained with ACA (data not shown), though to a lesser extent than did the nuclear spots produced by expression of GFP:HDaxx1-740 (Fig. 7I-L). The decreased centromere colocalization of GFP:HDaxx573-740 (which contains the CENP-C-interacting domain of HDaxx) may, in part, be due to improper conformation of this partial HDaxx peptide, which contains the 27 kDa GFP moiety fused to its amino terminus. Alternatively, efficient centromere localization of HDaxx may require that the carboxyl-terminal domain be physically coupled to the rest of the protein.

The distribution of GFP:HDaxx1-573 in live transfected cells was predominantly nuclear though a variable amount of cytoplasmic fluorescence was always apparent (Fig. 9C,D). Some cells expressing GFP:HDaxx1-573 also displayed nuclear spots (Fig. 9D) which we found did not colocalize with interphase centromeres detected with human ACA (data not shown). Thus, HDaxx lacking the carboxyl-terminal residues that interact with CENP-C in yeast accumulates in non-centromeric nuclear spots. The nature of these spots, and whether they correspond to the non-centromeric spots observed by staining cells for endogenous HDaxx remains to be
determined. In no case did we observe any obvious phenotypes, such as the induction of cell death or cell-cycle arrest, resulting from the over-expression of any of the GFP:HDaxx fusion constructs in human cells. Collectively, these results suggest that independent domains of HDaxx mediate its association with interphase centromeres and with other unknown nuclear substructures.

Fig. 8. HDaxx is present throughout the cell cycle, but its association with centromeres is restricted to interphase. Endogenous HDaxx and intrinsic centromere proteins were simultaneously localized by indirect immunofluorescence detection in: (A-C) a mitotic HeLa cell in prophase, (D-F) a mitotic HeLa cell in metaphase, and (G-I) an interphase HeLa cell in G1 or early S phase. (A,D,G) DNA staining by DAPI; (B,E,H) HDaxx staining by anti-HDaxx; (C,F,I) centromere staining by human autoimmune serum GS. HDaxx is present throughout the cell cycle at a constant steady state level. (J) The 116 kDa region of an immunoblot of probed with anti-HDaxx in which each lane contains 30 µg total protein from HeLa cell populations synchronized in the cell cycle by mitotic shake-off (Monteiro and Mical, 1996). M, mitotic cells; numbers refer to the time, in hours, when protein extracts were prepared after mitotic cells collected by shake-off were plated; Ex, exponentially growing cells. Bars, 10 µm.

Fig. 9. Localization of GFP and GFP:HDaxx fusion proteins in live HeLa cells. The distribution of GFP and GFP:HDaxx fusion proteins was observed by fluorescence microscopy of live HeLa cells transfected with (A) the empty GFP vector, pEGFP-C1, (B) pGFP:HDaxx1-740, (C,D) pGFP:HDaxx1-573, and (E) pGFP:HDaxx573-740. (F) Immunoblot of total protein lysates collected from cell populations transfected with lane 1, pEGFP-C1; lane 2, pGFP:HDaxx1-740; lane 3, pGFP:HDaxx1-573; lane 4, pGFP:HDaxx573-740. GFP and GFP:HDaxx fusion proteins were detected with an antibody specific for GFP. Molecular mass standards (in kDa) are indicated. Bar, 10 µm.
CENP-C binding protein, HDaxx

Immunoblot analysis of whole cell lysates collected from transfected HeLa cell populations revealed that all constructs expressed GFP fusion peptides of the expected sizes (Fig. 9F). Interestingly, GFP:HDaxx 573-740 expressed in HeLa cells was always detected as a broadly migrating band on immunoblots, suggesting that the carboxyl terminus of HDaxx may be a target for post-translational modification in vivo.

DISCUSSION

The human centromere is a dynamic chromosomal structure that undergoes striking morphological and functional transformations throughout the cell cycle, the best-characterized of which are accompanied by its transient association with specific proteins during mitosis. Here we report the unexpected interphase-specific association of human centromeres with HDaxx, a protein whose murine homolog was previously identified through its ability to bind the death domain of the cell surface receptor Fas (CD95). The association of HDax with interphase centromeres is mediated by its interaction with the amino terminus of the intrinsic centromere protein CENP-C, with which it interacts in the yeast interaction trap assay. Targeting of HDax to interphase centromeres is determined by residues in its carboxyl terminus, which are also responsible for its interaction with CENP-C and with the intracellular death domain of Fas. These results suggest a previously unsuspected role for interphase centromeres in the regulation of cellular responses to death stimuli.

CENP-C is one of the few known proteins that colocalizes with centromeric heterochromatin at all stages of the cell cycle. This juxtaposition is best characterized in condensed mitotic chromosomes, where CENP-C is found in the inner kinetochore plate (Saitoh et al., 1992). While the structural and functional status of centromeres during interphase is less clear, the presence of CENP-C at interphase centromeres is nevertheless important for proper kinetochore structure and function in the subsequent mitosis. This was first demonstrated by antibody microinjection studies, which showed that the structural integrity of the kinetochore as well as mitotic progression were disrupted when antibodies to CENP-C were injected into interphase cells, but not when they were injected into mitotic cells (Tomkiel et al., 1994). Because little or no CENP-C could be detected in the antibody-injected cells, it was suggested that these effects resulted from depletion of CENP-C from interphase centromeres, indicating that interphase centromeres are not functionally quiescent at this point in the cell cycle.

Similar results were obtained in a chicken cell line containing a conditional mutation in the cognate chicken gene for CENP-C that causes the efficient removal of CENP-C from centromeres and results in a complete block in the metaphase to anaphase transition (Fukagawa and Brown, 1997). Interestingly, these mitotically arrested cells subsequently died by apoptosis. Targeted disruption of the mouse CENP-C gene was also recently reported to result in disruption of mitosis and early embryo death (Kalitsis et al., 1998). The apoptotic response to the loss of CENP-C in the chicken cells suggests the perturbation of a regulatory function that links mitotic progression with cell viability. While it was argued that CENP-C is not necessary for the cell to successfully traverse interphase, the resulting metaphase block is nonetheless consistent with an important function, or perhaps interaction, that must occur in interphase but is not manifest until the subsequent mitosis. How such functions are controlled so they are not expressed inappropriately is not known, but it is reasonable to presume that cell cycle-specific protein-protein interactions and/or protein modifications could be involved.

Although indirect, three lines of evidence suggest that HDaxx, which interacts with CENP-C and colocalizes with human interphase centromeres, is the functional human homolog of mouse Daxx, a novel signaling protein that enhances Fas-mediated apoptosis by activating the Jun N-terminal kinase pathway. First, the carboxyl-terminal 112 amino acids of both mouse Daxx and HDaxx interact strongly with mouse Fas intracellular region, and the carboxyl terminus of HDaxx also interacts with human FasIC (Yang et al., 1997). Second, the two proteins are 73% identical, and nucleotide sequence conservation enabled the mouse Daxx cDNA to be cloned by hybridization with the 3’ end of the human Daxx cDNA (Yang et al., 1997). Finally, the most compelling evidence for functional conservation between HDaxx and mouse Daxx was the demonstration that overexpression of the carboxyl terminus of mouse Daxx with Fas suppressed Fas-mediated apoptosis in HeLa cells, a dominant-negative effect that was reversed when full-length mouse Daxx, the carboxyl terminus of mouse Daxx and Fas were co-expressed in HeLa cells, suggesting that the carboxyl terminus of the mouse protein competed with an endogenous human protein (presumably HDaxx) for binding to Fas (Yang et al., 1997).

We have demonstrated by biochemical fractionation and by immunolocalization that the majority, and possibly all, of endogenous HDaxx is found in the nuclei of human tissue culture cells. This result is consistent with HDaxx’s interaction with CENP-C and its colocalization with human interphase centromeres, but it presents a paradox if HDaxx and mouse Daxx are true functional homologs, since transduction of apoptotic signals by Daxx is thought to occur through its direct binding to the cytoplasmic death domain of Fas at the plasma membrane (Yang et al., 1997). However, the lack of direct evidence for cell-surface localization of mouse Daxx as well as the presence of three putative nuclear localization signals within the mouse Daxx amino acid sequence raises the possibility that endogenous mouse Daxx, like HDaxx, also normally resides in the nucleus. We can envision two scenarios by which HDaxx could gain access to the cytoplasm to bind cell surface death domains. First, the interaction of HDaxx with Fas death domains could occur by default after nuclear envelope breakdown at prometaphase when, as we have shown, HDaxx is present in the cell but not associated with centromeres or confined to the nuclear compartment. Alternatively, HDaxx may shuttle between the nucleus and cytosol, perhaps in response to activation of Fas. Kiriakidou et al. (1997) recently reported that an epitope-tagged protein essentially identical to HDaxx was detected in both the nucleus and cytoplasm when over-expressed in COS cells. Although we cannot completely rule out the possibility that cytoplasmic HDaxx exists in human cells or that a minor pool becomes cytoplasmic when over-expressed, we have seen no evidence for significant amounts of full-length HDaxx in the cytoplasm of either transfected or untransfected HeLa cells.

It is curious that essentially the same region of HDaxx has been found to interact with CENP-C and Fas, two proteins that share no recognizable common features and that reside in different subcellular compartments. It is possible that subdomains within
the carboxyl terminus of HDaxx have unique binding specificities whose activities are differentially regulated in the cell. In this regard, our demonstration that the carboxyl terminus of HDaxx appears to be a target for post-translational modification may provide a mechanism for the regulation of HDaxx interaction with CENP-C and/or Fas. Although there is evidence indicating that HDaxx can modulate the apoptotic response under certain circumstances, conflicting reports question the generality of a parallel Daxx-mediated apoptotic pathway downstream of Fas that is distinct from the FADD-mediated pathway (Yang et al., 1997; Zhang et al., 1998; Wajant et al., 1998). Thus, our demonstration that HDaxx is a nuclear protein and that it transiently associates with interphase centromeres suggests that its roles in vivo may be more complex than previously suspected.

The interphase centromere has been a notoriously elusive subject for study not only because it resides in a structurally undistinguished collection of chromatin in the nucleus, but because it is not well-characterized biochemically. ACA localization and fluorescence in situ hybridization studies have indicated that the only subcellular structures with which interphase centromeres routinely associate are the nuclear envelope and nucleoli (Brenner et al., 1981; Moroi et al., 1981). In a previous search for nuclear proteins that interact with CENP-C, we found that the nucleolar transcription factor UBF/NOR-90 specifically binds the carboxyl terminus of CENP-C (Pluta et al., 1995). While the functional significance of that interaction remains unclear, it is consistent with the ultrastructural and biochemical detection of human CENPs in isolated nucleoli and supports the argument that the dominant autoantigen in scleroderma spectrum disease is a macromolecular complex consisting of centromeres and nucleoli (Tan et al., 1988; Ochs and Press, 1992). It was recently reported that proteins encoded by two genes associated with early-onset familial Alzheimer’s disease, PS1 and PS2, colocalize with centromere antigens recognized by human ACA in interphase nuclei (Li et al., 1997). It is not known which, if any, of the intrinsic CENPs are involved in this colocalization.

Our identification of a protein that associates with an intrinsic component of the centromere specifically during interphase provides not only a unique opportunity to functionally characterize interphase centromeres, but a potential link between centromeres and a cell death pathway. If HDaxx, like mouse Daxx, potentiates Fas-mediated apoptosis through direct binding of a cell surface death receptor, centromere association during interphase may serve a cell cycle-regulated sequestering function to control cellular responses to apoptotic stimuli. Thus, perhaps in addition to sensing spindle damage during mitosis by their transient association with HDaxx in interphase. Alternatively, HDaxx may have additional/separate functions within the nucleus, perhaps linking chromosomal events during interphase with the Jun-N-terminal kinase signalling pathway that is involved in cell cycle regulation rather than the apoptotic response.

We thank Dr R. Brent for generously providing the yeast two-hybrid reagents; Dr J. Tomkiel for DNA constructs; Dr J. Boeke for E. coli JBE15; Dr M. Monteiro for providing protein lysates from synchronized HeLa cells; and A. Cox and L. Ostrowski for excellent technical assistance. A.F.P is grateful to Drs M. Monteiro, L. Casiola-Rosen and A. Rosen for their support and encouragement during different stages of this work. This work was supported in part by an Arthritis Investigator Award to A.F.P. The GenBank accession number for the sequence of HDaxx reported in this paper is AF050179.

REFERENCES


