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SHORT NOTE

The Nucleoporin CAN/Nup214 Binds to both the Cytoplasmic and the Nucleoplasmic Sides of the Nuclear Pore Complex in Overexpressing Cells

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CAN/Nup214, an essential component of the vertebrate nuclear pore complex (NPC), is required for proper cell cycle progression and nucleocytoplasmic transport. It is a member of the FG-repeat-containing family of nucleoporins and has been localized to the cytoplasmic face of the NPC. Indirect immunofluorescence studies with specific antibodies have shown that moderate overexpression of human CAN in HeLa cells causes an increase in CAN/Nup214 levels at the nuclear envelope. Here, we demonstrate that in such HeLa cells, CAN/Nup214 does not localize exclusively to the cytoplasmic side of the NPC. Cryosections, stained with CAN-specific antibodies and examined by electron microscopy, showed that about one-third of the gold-labeled NPCs were decorated at the cytoplasmic face and the remaining two-thirds at the nucleoplasmic face. These data indicate that both the cytoplasmic fibrils and the nuclear basket of the vertebrate NPC contain specific binding sites for either CAN/Nup214 or for its interacting proteins, Nup88 and hCRM1. Thus, it is conceivable that CAN/Nup214 functions in nucleocytoplasmic transport at both faces of the NPC.

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INTRODUCTION

The vertebrate nuclear pore complex (NPC), an ~125 MDa multiprotein assembly that mediates bidirectional transport between the cytoplasm and the nucleus, is composed of a spoke complex embracing a central channel or transporter. A cytoplasmic ring with filaments emanating into the cytoplasm is attached to

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the spoke complex, as is a nuclear ring with filaments that converge to form the nuclear basket [for reviews see 1, 2]. The NPC consists of multiple copies of an estimated 100 different proteins (nucleoporins), including integral membrane proteins and peripheral proteins. A number of these nucleoporins contain multiple degenerate peptide motifs that share an FG dipeptide core sequence [reviewed by 1, 3–5].

Protein import into the nucleus is mediated by a heterodimer comprising the nuclear localization signal (NLS)-receptor/importin α , which recognizes the NLSs of karyophilic proteins, and nuclear import factor p97/importin β , which contacts the NPC [reviewed by 6; see also 7–9]. After this first docking step, the ligand complex is imported via an energy-dependent process that requires the small GTPase Ran/TC4 [10, 11]. GTP hydrolysis by Ran is thought to drive the translocation reaction [12–14], which may involve repeated association and dissociation of import complex components to the NPC [15, 16], or the movement of a single complex across the NPC either in discrete steps [17] or in a sliding motion [9]. Many FG-repeat-containing nucleoporins are located at distinct positions along the NPC and may form binding sites for transport complexes [15, 18, 19]. Protein and ribonucleoprotein (RNP) export from the nucleus is also a receptor-mediated, energy-dependent process [for reviews see 8, 20], and involves some of the same factors necessary for protein import [17].

The FG-repeat-containing nucleoporin CAN, also called Nup214 and p250, is detected exclusively at the cytoplasmic face of the NPC in HeLa cells [21]. In *Xenopus* oocytes CAN was sublocalized to the cytoplasmic filaments of the NPC [22]. Recently, we made a knock-out mutation of the mouse CAN gene. Mouse embryos that lack CAN show growth arrest, followed by simultaneous defects in polyadenylated RNA export and protein import [23]. Further, coimmunoprecipitation ex-

periments demonstrated that CAN is part of a complex with at least two other polypeptides—one of 88 kDa and the other of 112 kDa [24]. Recently, these proteins were identified as the novel NPC component Nup88 and hCRM1, a homolog of yeast CRM1 that is localized to the NPC and nucleoplasm [25].

In previous studies, we found that overexpression of CAN in HeLa-derived cells results in an increase of this nucleoporin at the nuclear envelope [26]. To examine the sublocalization of overexpressed CAN within the NPC, we have used ultrathin cryosections in combination with immunogold labeling techniques.

MATERIALS AND METHODS

Immunoelectron microscopy. HeLa-derived cell line CE490S has been described previously [26]. Cells were grown in the presence or in the absence of 1 μ g/ml tetracycline, fixed with 2% paraformaldehyde in bicarbonate buffer (pH 7.4) for 90 min at room temperature, and stored in 1% paraformaldehyde in phosphate buffer (pH 7.3) at 4°C. Cells were scraped from the dish and pelleted in 10% gelatin. Ultrathin cryosectioning and immunolabeling were performed as described before [27]. Sections (~100 nm) were incubated with polyclonal α CNN antiserum [26], followed by protein A complexed to 10-nm gold particles, before being examined and photographed by using a JEOL JEM1010 electron microscope, operating at 80 kV.

RESULTS

CE490S cells can be induced to overexpress CAN because they contain the CAN cDNA, under the control of the tet-operator promoter [26, 28]. In the presence of 1 μ g/ml tetracycline, CE490S cells express endogenous levels of CAN. However, if tetracycline is removed from the medium, CAN is moderately overexpressed, without detectably affecting cell growth or nuclear envelope structure. Indirect immunofluorescence studies using our polyclonal α CNN antiserum, which is directed against unique sequences in the N-terminus of CAN, showed that the protein is located mainly at the nuclear envelope in cells expressing normal CAN levels and in cells overexpressing CAN [26].

For ultrastructural localization studies, ultrathin cryosections of CE490S cells were first incubated with α CNN, followed by protein A complexed to 10-nm gold particles. In CAN-overexpressing cells, gold particles decorated both cytoplasmic and nucleoplasmic sides of the NPCs (Fig. 1). No significant label was found in the cytoplasm or nucleoplasm, indicating that the overexpressed protein specifically associated with the NPCs. Quantitation of the gold distribution in 100 nuclei showed that 59% of the NPCs were decorated at the nucleoplasmic side (Fig. 1B), 36% at the cytoplasmic side (data not shown), and 5% at both sides or in the middle (Fig. 1A). The amount of label detected at both sides of the NPCs varied, which is probably due to the plane of section. Our antibody did not detect gold label

in CE490S cells expressing endogenous levels of CAN (data not shown).

DISCUSSION

Our immunoelectron microscopic studies on CAN-overexpressing HeLa cells showed that CAN localized to both the cytoplasmic and the nucleoplasmic sides of the NPC. This result indicates that specific binding sites for CAN, or for its interacting proteins, exist on both faces of the nuclear pore. The NPCs of normal HeLa cells did not stain, which demonstrates that immunoelectron detection of CAN with our antiserum, directed against N-terminal CAN sequences, requires elevated levels of the protein. In previous immunolocalization studies CAN is detected only at the cytoplasmic face of NPCs of normal HeLa cells and *Xenopus* oocytes [21, 22]. Why is CAN found at the nucleoplasmic side of the NPC in overexpressing HeLa cells?

Studies on normal HeLa cells employed a specific antibody against C-terminal CAN sequences, which localized CAN to the cytoplasmic face of NPCs [21]. It is conceivable that epitopes in the C-terminal part of CAN are masked specifically at the nucleoplasmic side of the NPC. This region may for instance interact with certain proteins or protein complexes in the nucleus, thereby preventing antibody binding. On the other hand, the levels of endogenous CAN at the nucleoplasmic side may be too low to detect with the C-terminal CAN antiserum.

CAN has also been detected at the cytoplasmic fibrils of *Xenopus* oocyte NPCs with antibodies directed against the entire protein [22]. Interestingly, the FG-repeat-containing nucleoporin p62 is found exclusively at the nucleoplasmic face of NPCs of *Xenopus* oocyte nuclear envelopes [2, 29]; however, in mammalian liver cells, this protein localizes to both sides of the NPC [29, 30]. Consequently, the sublocalization of nucleoporins at the NPC may vary with cell-type or with the expression level of the nucleoporin.

Altered expression levels of nucleoporins may potentially disrupt the normal distribution or architecture of the NPCs. However, our electron micrographs showed no gross alterations of nuclear envelope structure, such as herniations or NPC clustering [see, e.g., 31, 32]. Moreover, the growth rate of CE490S cells was not affected, suggesting that moderately elevated CAN levels do not disrupt nucleocytoplasmic transport [33]. Finally, it cannot be absolutely excluded that even mild overexpression of CAN induces an artificial localization of the protein at the nuclear side of the NPC, without any relevance to the mechanism of nucleocytoplasmic trafficking. However, the specificity of the localization of overexpressed CAN protein is striking: CAN is exclusively detected at the NPCs, and not in any other cellular compartment or structure. This argues that CAN has the ability to bind

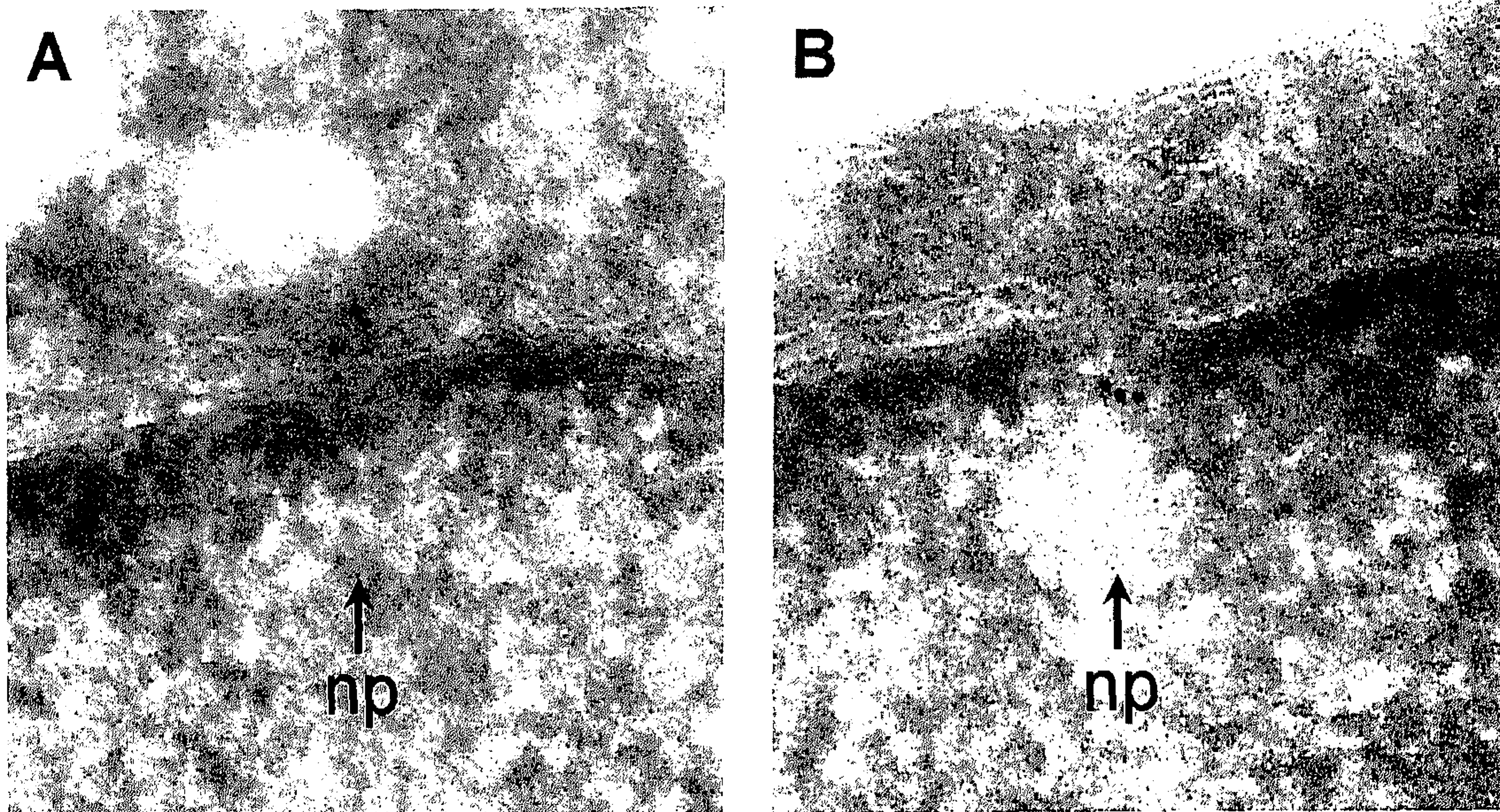


FIG. 1. CAN localizes to both sides of the NPCs of CAN-overexpressing CE490S cells. Ultrathin cryosections were immunolabeled with polyclonal α CNN antiserum, followed by 10-nm gold-conjugated protein A. Selected examples of labeled NPC cross sections are aligned with their cytoplasmic and nucleoplasmic sides up and down, respectively. The NPCs are indicated (np). (A) Localization of epitopes of N-terminal CAN at both the cytoplasmic and the nucleoplasmic sides of the NPC. (B) Gold decorating the nucleoplasmic side of the NPC. Magnification is 120,000 times.

specifically to nucleoplasmic components of the nuclear pore complex, in addition to its established association with the cytoplasmic fibrils, and that mildly overexpressing cells could very well reinforce a normal biological situation.

Repeat-containing NPC proteins, such as CAN, are believed to play an important role in transport across the NPC, but the precise function of the repeats in this process is unknown [4, 5]. To date, *in vitro* studies have shown that FG peptide motifs may serve as binding sites along the pore for specific factors carrying transport substrates [18, 19, 33, 34]. Each NPC protein that has been localized to a specific subregion of the NPC, with the exception of p62, is confined to a specific niche either on the cytoplasmic or the nucleoplasmic side. Immunoelectron microscopy has demonstrated that RanBP2/Nup358 localizes to the cytoplasmic fibrils [21, 22, 35–37], whereas Nup153 and Nup98 are constituents of the nuclear basket [18, 22, 38, 39]. These findings have provided the basis for several transport models in which FG-repeat-containing nucleoporins form a static array of docking sites that transiently interact with transport complexes [8, 9, 15].

Based on our subcellular localization data it appears that CAN could interact with transport complexes at both sides of the NPC. CAN depletion in mouse embryos results in defects in both nuclear protein import and mRNA export. Therefore, a symmetric distribution of CAN may be important for bidirectional transport of different substrates through the nuclear pore. Like

CAN, other nucleoporins could also localize to multiple sites within the NPC. In this respect, it is interesting that Cordes *et al.* [39] found a small but significant amount of Nup153 at the cytoplasmic side of the NPC of mouse liver cells and *Xenopus* nuclei that was not detected in studies where harsher fixation conditions were used. Alternatively, the presence of CAN at the cytoplasmic and nucleoplasmic fibrils could reflect CAN's ability to move through the pore with the transport complex.

It will be interesting to assess whether overexpressing other NPC proteins affects their sublocalization. The finding that some nucleoporins are present in different subregions of the NPC at distinct concentrations may be relevant to our understanding of the role of nucleoporins in nucleocytoplasmic transport.

We thank Maarten Fornerod for discussions and for critically reading the manuscript, Sjožef van Baal for excellent technical assistance, Dr. Sue Vallance for scientific editing, and Peggy Burdick for secretarial assistance. These studies were supported in part by Cancer Center CORE Grant CA-21765 and by the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital.

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Received October 22, 1996

Revised version received January 21, 1997