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Technical Note

Ectopic expression of plasma membrane targeted subunits of the Ndc80-complex as a tool to study kinetochore biochemistry

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ABSTRACT

Genomic stability depends on the normal function of the kinetochore, a multi-protein assemblage, which consists of over 80 molecules including both constitutive and transiently binding components. Information regarding the spatial-temporal assembly of kinetochore subcomplexes is often limited by technical difficulties in their isolation. To study kinetochore subcomplex formation, we targeted separately Hec1 and Spc24, two subunits of the Ndc80 kinetochore compilation, to the plasma membrane by fusing them with the amino-terminal palmitoylation and myristoylation (pm) sequence of the receptor tyrosine kinase Fyn. We found that in early mitotic cells, pm-GFP-Hec1 and pm-GFP-Spc24 fusion proteins localised to the plasma membrane and were able to recruit all subunits of the Ndc80 complex (Ndc80/Hec1, Nuf2, Spc24 and Spc25) to these foci. In interphase cells, only Hec1-Nuf2 and Spc24-Spc25 heterodimers accumulated to the plasma membrane foci. The results propose that the assembly of Ndc80 tetramer can take place outside of the kinetochore but requires co-factors that are only present in mitotic cells. These findings provide the first experimental evidence on the successful employment of the plasma membrane targeting technique in the study of kinetochore biochemistry.

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1. Introduction

In cell division, the accurate delivery of chromosomes between the two daughter cells is dependent on many kinetochore coordinated processes including the establishment and maintenance of correct microtubule–chromosome attachments, chromosome motility, and the timing of

chromosome segregation. The vertebrate kinetochore, referring here to the trilaminar plate structure formed on the centromeric region of chromatin, consists of over 80 different proteins (Chan et al., 2005; Cheeseman and Desai, 2008). Some of these proteins are constitutive components that are present throughout the cell cycle, whereas others are transient co-factors and regulatory molecules that accumulate to

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kinetochores at specific time points in mitosis (Maiato et al., 2004; Musacchio and Salmon, 2007). Recent studies have highlighted the importance of kinetochore protein dynamics in the regulation of the spindle checkpoint, a conserved signalling cascade that monitors the fidelity of chromosome segregation (Musacchio and Hardwick, 2002; Kops, 2008). Individual kinetochores can rapidly alter their biochemical protein identity in response to changes in chromosome positioning and microtubule attachment status, and thereby control the molecular switches that regulate spindle checkpoint activity. Errors in kinetochore protein function may result in gain or loss of one or more chromosomes (aneuploidy), which can lead to malignant cell transformation or cell death (Chi and Jeang, 2007).

The study of kinetochore biochemistry is hampered by the lack of tools for the isolation of kinetochore subcomplexes with spatial-temporal resolution. To overcome this limitation, researchers have used kinetochore assembly assays in *Drosophila melanogaster* (Przewlaka et al., 2007) and in *Xenopus* egg extracts *in vitro* (Desai et al., 1997). Despite these efforts, it is still largely unknown when and where the different kinetochore complexes form in mammalian cells. One attractive method for the study of kinetochore complex assembly is the plasma membrane targeting technique where selected proteins are directed to the membrane by fusing them with a known plasma membrane localisation sequence. In general, three different signals can facilitate the membrane association: a myristate with a basic signal, myristate plus palmitate, or two palmitates. The N-terminal motif of a protein containing any of these signals can be used as added cassette in other proteins to confer membrane binding. The target fusion proteins localise to plasma membrane in an orientation, in which the membrane-targeting sequence is embedded in the inner lipid layer of the membrane and the fusion protein with a -possible tag is in the cytoplasm. Fusion proteins with membrane-targeting signals have been employed in several types of applications. The fatty acylation motifs have been shown to confer targeting of proteins to specific plasma membrane microdomains, such as rafts or caveolae enriched in glycosphingolipids and cholesterol (Zhou et al., 1994). In addition, many cytosolic signalling molecules are known to localise to the inner face of the plasma membrane and this has enabled the use of the technique in functional studies on signal transmission involving the recruitment of soluble proteins to membrane-bound receptors independent of receptor activation (Galbiati et al., 1999). Fatty acylation motifs have also been used to replace or circumvent existing membrane-targeting signals within a given protein and in analyses of post-translational modifications in signal transmission (Seykora et al., 1996). Finally, chimaeric proteins have been employed to test the membrane-targeting potency of a given sequence as, for example, in studies where the attachment of the N-terminal sequences of Fyn or inhibitory G protein to the soluble carrier protein resulted in fusion protein targeting to detergent-resistant membrane subdomains (van't Hof and Resh, 1997; Galbiati et al., 1999).

Here we have used plasma membrane targeting of mitotic proteins as an approach to distinguish kinetochore complexes that are formed at the kinetochore from complexes that are formed outside of the kinetochore. To this end, we have

targeted Hec1 and Spc24, two subunits of the conserved Ndc80 complex, to the plasma membrane and investigated whether they are able to recruit other Ndc80 complex proteins to the same plasma membrane foci in interphase and M-phase cells. Ndc80 complex is a tetramer composed of single copies of four subunits, namely Ndc80 (Hec1 in mammals), Nuf2, Spc24 and Scp25 (Ciferri et al., 2005; Wei et al., 2005). *In vitro*, the subunits pair to form stable Hec1–Nuf2 and Spc24–Scp25 heterodimers, which can associate with each other and organise into a rod-like dumbbell-shaped tetramer comprising the full Ndc80 complex. The Ndc80 complex localises between the inner and outer kinetochore plates where it functions to facilitate proper microtubule–kinetochore attachments and spindle checkpoint signalling (McClelland et al., 2004; Ciferri et al., 2005; Wei et al., 2005; DeLuca et al., 2006; Dong et al., 2007; Miller et al., 2008). The Ndc80 complex is a relatively stable component of the mitotic kinetochore as Hec1 and Nuf2 bind to this structure with half-times of several minutes (Hori et al., 2003). Moreover, our earlier study shows that the complex localises to kinetochores in mitosis while no kinetochore accumulation is observed in interphase (McClelland et al., 2003). When overexpressed in chicken DT40 cells, Ndc80 and Nuf2 fusion proteins localise to centrosomes in G1 and S phase and to kinetochores in G2/M phase, respectively (Hori et al., 2003). However, the details of Ndc80 complex assembly are poorly understood.

2. Results and discussion

To re-localise the chosen kinetochore proteins, Hec1 and Spc24, to the plasma membrane, we have used a known plasma membrane localisation sequence consisting of the N-terminal palmitoylation and myristoylation sequence of the receptor tyrosine kinase Fyn (N–pmFyn, McCabe and Berthiaume, 1999, 2001). The N–pmFyn has been shown to efficiently target GFP to the plasma membrane (McCabe and Berthiaume, 1999). We cloned GFP-tagged N–pmFyn fusions of well characterised kinetochore proteins Hec1 (pm-GFP–Hec1) and Spc24 (pm-GFP–Spc24). The N–pmFyn consisted of one myristate and two palmitates fused with a GFP-tag (Figure 1A). The correct sizes of pm-GFP, pm-GFP–Hec1 and pm-GFP–Spc24 were verified by Western blot analyses (Figure 1B). Analysis of cells transiently transfected with either pm-GFP–Hec1 or pm-GFP–Spc24 demonstrated that both fusion proteins localised to the plasma membrane in interphase cells (Figure 1C). In mitotic cells, pm-GFP–Hec1 and pm-GFP–Spc24 showed accumulation at distinct foci at the plasma membrane (Figure 1C). In addition to this, pm-GFP–Spc24 and especially pm-GFP–Hec1 exhibited intense kinetochore signals (Figure 1C) corresponding to the localisation of endogenous Hec1 and Spc24 during mitosis (Figure 1D). This observation was unexpected as the N–pmFyn signal was presumed to target the whole fusion protein pool to the plasma membrane. A plausible explanation is that one of the Ndc80 complex subunits, or a binding partner carrying a specific kinetochore localisation signal, targeted a portion of the fusion protein pool to kinetochores. Candidate co-factors include the KNL1/Spc105 and the Mtw1/Mis12 complex that together with the Ndc80 complex form a stoichiometric protein assembly

Figure 1 – Plasma membrane localisation of pm-GFP-Hec1, pm-GFP-Spc24 and pm-GFP. (A) Schematic pictures of pm-GFP-Hec1, pm-GFP-Spc24 and pm-GFP fusion proteins. (B) Western blot of HeLa cells expressing pm-GFP-Hec1, pm-GFP-Spc24 and pm-GFP fusion proteins. (C) Localisation of pm-GFP-Hec1, pm-GFP-Spc24 and pm-GFP in the HeLa cells at interphase (upper row) and mitosis (lower row). In the interphase cells, portions of the fusion proteins accumulate to the plasma membrane (arrowheads), punctuate foci in the cytosol (arrows) and some remain soluble in the cytoplasm. In the mitotic cells, the fusion proteins accumulate to the plasma membrane (arrowheads), kinetochores (arrows) and some remain in the cytosol. (D) Localisation of the endogenous Hec1 and Aurora B at the outer kinetochores and inner centromeres of a mitotic HeLa cell, respectively. The linescan shows Hec1 and Aurora B signal intensities across a sister kinetochore pair. DNA was stained with DAPI. Bars: 10 μ m.

known as the KMN network (for KNL1, Mtw1/Mis12 complex, and Ndc80, [Cheeseman et al., 2006](#)). Alternatively, a fraction of the pm-GFP-Hec1 pool may be subjected to post-translational modifications that prevent the proper function of the N-pmfyn.

To test if plasma membrane targeted Ndc80 complex subunits are able to recruit other members of the complex to the same foci, we co-expressed pm-GFP, pm-GFP-Hec1 or pm-GFP-Spc24 with dsRed-monomers of Hec1, Nuf2, Spc24 and Spc25 and analysed their localisation using fluorescence microscopy. We first looked at complex assembly in interphase cells. As expected, targeting pm-GFP to the plasma membrane did not result in the recruitment of any dsRed monomers ([Figure 2A](#)). However, pm-GFP-Hec1 was able to recruit dsRed-Nuf2 to the plasma membrane but dsRed-Spc24 and dsRed-Spc25 were recruited to a much lesser extent ([Figure 2B](#)). Similarly, pm-GFP-Spc24 recruited specifically

dsRed-Spc25 to the plasma membrane but not dsRed-Hec1 or dsRed-Nuf2 (data not shown). Importantly, pm-GFP-Hec1 and pm-GFP-Spc24 were unable to recruit dsRed-Hec1 or dsRed-Spc24 to the plasma membrane, respectively ([Figure 2B](#)). This was in accordance with previous reports demonstrating that the Ndc80 complex is a tetramer composed of single subunit copies ([McClelland et al., 2004](#); [Wei et al., 2005](#); [Ciferri et al., 2005](#)). Our data showing that Hec1-Nuf2 and Spc24-Spc25 heterodimers can assemble in interphase cells suggests that cofactors that are needed for the heterodimerisation, if any required, are available throughout the cell cycle.

Next we studied the formation of the Ndc80 complex in mitosis. pm-GFP, pm-GFP-Hec1 or pm-GFP-Spc24 was expressed with dsRed-Hec1, -Nuf2, -Spc24, or -Spc25, and the localisation of the fusion proteins was analysed in early mitotic cells using fluorescence microscopy ([Figure 3](#)). In accordance with

Figure 2 – pm-GFP-Hec1 recruits Nuf2 to the plasma membrane in interphase cells. (A) Interphase HeLa cells co-expressing pm-GFP with dsRed-Hec1, –Nuf2, –Spc24 or –Spc25. None of the dsRed monomers accumulate at the plasma membrane with the pm-GFP (arrowheads). (B) Interphase HeLa cells co-expressing pm-GFP-Hec1 with dsRed-Hec1, –Nuf2, –Spc24 or –Spc25. dsRed-Nuf2 shows accumulation to same plasma membrane foci where pm-GFP-Hec1 resides (arrowheads) whereas no co-localisation is observed between pm-GFP-Hec1 and dsRed-Hec1, –Spc24 or –Spc25. Bars: 10 μ m. Merge shows overlay of the first two images in each row (green: GFP; red: Hec1, Nuf2, Spc24 or Spc25).

the analysis of interphase cells, targeting pm-GFP to the plasma membrane in mitotic cells did not lead to the recruitment dsRed-monomers (Figure 3A). Similarly to the behaviour of the endogenous protein, a large portion of all dsRed-Ndc80 complex monomers accumulated to kinetochores in prometaphase and metaphase cells, and a fraction remained soluble in the cytosol (Figure 3A). Importantly, pm-GFP-Hec1 was able to recruit a portion of dsRed-Nuf2, –Spc24 and –Spc25 monomers to the plasma membrane in mitosis (Figure 3B). Similarly, pm-GFP-Spc24 recruited portions of the dsRed-Spc25, –Hec1 and –Nuf2 monomers to the plasma membrane (data not shown). In agreement with the data from interphase cells, in mitosis pm-GFP-Hec1 did not recruit dsRed-Hec1 (Figure 3B) and pm-GFP-Spc24 did not recruit dsRed-Spc24 to the membrane (data not shown). Importantly, pm-GFP-Spc24 was able to recruit endogenous Hec1 (Figure 3C) and Nuf2 (data not shown) to the plasma membrane confirming that the re-localisation is not an artefact caused by protein overexpression. Finally, as expected, pm-GFP did not result in the transfer of endogenous Hec1 to the plasma membrane (Figure 3C).

In summary, our results show that a notable portion of three dsRed-Ndc80 complex monomers co-localise with the fourth N-pmFyn conjugated subunit at the plasma membrane in early mitotic cells. This proposes that the entire Ndc80 complex can form outside of the kinetochore in mitotic cells. Interestingly, pm-GFP-Hec1-Nuf2 and pm-GFP-Spc24-Spc25 heterodimers were formed in interphase cells but they did not assemble into Ndc80 complex tetramers, suggesting that the assembly of the full complex requires cofactors and/or post-translational modifications that are specific to the mitotic phase. Based on these results we propose a model for the forced Ndc80 complex formation at the plasma membrane (Figure 4). Our results

demonstrated that the plasma membrane targeting of kinetochore proteins is a potential tool to study the assembly of kinetochore protein complexes with spatial-temporal resolution. The technique could be used to study, for example, the minimal requirement for the capture of microtubules by a kinetochore subcomplex carrying a N-pmFyn conjugate (Figure 4). However, the fact that the N-pmFyn conjugated protein of interest is stably bound to the plasma membrane may limit the utilisation of the method for kinetochore complexes whose assembly is not dependent on high turnover rate of the N-pmFyn conjugated subunit or on co-factors stably bound at the kinetochores. The future applications of the method may include combinations with the proximity ligation technique (Gustafsdottir et al., 2005) and isolation of the membrane fractions for biochemical analyses.

3. Experimental procedures

3.1. Cell culture and transfection

HeLa and HT-1080 cells were obtained from ATCC. All cells were cultured at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated foetal calf serum (FCS; Invitrogen) and penicillin-streptomycin (100 IU/ml and 100 mg/ml respectively). For transient transfections, subconfluent HeLa and U2OS cells were transfected with the fusion proteins or empty GFP control using FuGENE (Roche Applied Science, Indianapolis, IN) or Effectene (Invitrogen). Twenty-four hours

after transfections the cells were either processed for immunofluorescence or Western Blotting analyses.

3.2. Cloning

pcDNA3.1-pm-GFP (pm-GFP) containing the following amino acid sequence of Fyn; GCVQCKDKEA, was cloned from pCMV5-pm-GFP (McCabe and Berthiaume, 1999). Appropriate oligonucleotides containing NheI and HindIII restriction endonuclease recognition sites at the 5' and 3' ends, respectively, pCMV5-pm-GFP and Phusion DNA polymerase (Finnzymes, Espoo, Finland) were used in the PCR reactions. Amplified fragments were digested with corresponding restriction enzymes, gel purified and ligated into the appropriately digested pcDNA3.1/Zeo+ mammalian expression vector (Invitrogen). pcDNA3.1-pm-GFP-Hec1 (pm-GFP-Hec1) was cloned as follows: wild type Hec1 was digested from pEYFP-Hec1 using the HindIII and EcoRV restriction sites and ligated into HindIII and SmaI cleaved pcDNA3.1-pm-GFP. pcDNA3.1-pm-GFP-Spc24 (pm-GFP-Spc24) was cloned as follows: wild type Spc24 was digested from pEYFP-Spc24 using the HindIII and BamHI restriction sites and ligated into HindIII and BamHI cleaved pcDNA3.1-Zeo-pm-GFP. All constructs were verified to be correct by sequencing.

3.3. Immunofluorescence microscopy

Immunostaining of cells grown on coverslips was performed by simultaneous fixation and permeabilisation in 20 mM PIPES pH 6.8, 4% formaldehyde, 0.2% Triton X-100, 10 mM EGTA, 1 mM MgCl₂, for 10 min at room temperature. All fixed cells were incubated for 30 min at room temperature in blocking solution (PBS, 3% FCS). Primary antibodies used were a goat polyclonal GFP (Abcam, Cambridge, UK), mouse monoclonal Hec1 (Abcam), and mouse monoclonal Nuf2 (Sigma-Aldrich, St Louis, MO). Primary antibodies were detected with Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). All antibody incubations were carried out for 90 min at room temperature. Wide-field fluorescence microscope images were collected using an inverted Axiovert 200M stand (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) equipped with a Plan-Apochromat 63×/1.4 oil objective and Hamamatsu Orca ER CCD camera (Hamamatsu Photonics Norden, Solna, Sweden). Images were prepared by using the Metamorph imaging software (Molecular Devices, Downingtown, PA).

Figure 3 – pm-GFP-Hec1 recruits Nuf2, Spc24 and Spc25 to the plasma membrane in mitotic cells. (A) Early mitotic HeLa cells co-expressing pm-GFP with dsRed-Hec1, –Nuf2, –Spc24 or –Spc25. No accumulation of dsRed monomers is observed at the plasma membrane pm-GFP foci (arrowheads). **(B)** Early mitotic HeLa cells co-expressing pm-GFP-Hec1 with dsRed-Hec1, –Nuf2, –Spc24 or –Spc25. All dsRed monomers expect the dsRed-Hec1 co-localise with pm-GFP-Hec1 at the plasma membrane (arrowheads). **(C)** HeLa cells expressing pm-GFP or pm-GFP-Spc24 were fixed and immunostained with anti-Hec1 antibodies. A fraction of the endogenous Hec1 protein is recruited to the plasma membrane in the pm-GFP-Spc24 (arrowheads) but not in the pm-GFP expressing metaphase cells. Bars: 10 μm. Merge shows overlay of the first two images in each row (green: GFP; red: Hec1, Nuf2, Spc24 or Spc25).

Figure 4 – A model for Ndc80 complex formation at the plasma membrane. The Hec1/Nuf2 and Spc24/Spc25 heterodimers form in the interphase cells prior to entry into mitosis. N-pmfyn sequence in either Hec1 or Spc24 drives the individual heterodimers to the plasma membrane. At the beginning of mitosis, the membrane-targeted pm-Hec1–Nuf2 and pm-Spc24–Spc25 heterodimers recruit the other Ndc80 subunits to the same plasma membrane foci. The method may be used to study, for example, the minimum membrane-targeted kinetochore protein composition required for microtubule capture. For simplicity, only one centrosome is depicted.

3.4. Immunoblotting

For immunoblotting transiently transfected HeLa cells were lysed in Laemmli sample buffer prior to SDS–PAGE and Western blotting using a goat-polyclonal GFP antibody and goat-HRP antibody (Jackson Immunoresearch).

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