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Regulation of Dynamin by Nucleoside Diphosphate Kinase

Radhakrishnan Narayanan[1,2] and Mani Ramaswami[2]

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Nucleoside diphosphate (NDP) kinase is required for multiple cellular functions, including cell growth, motility, and differentiation, and its loss is associated with pathologies including tumor metastasis. A recent study has revealed a previously unknown function for NDP kinase as positive regulator of dynamin, a GTPase essential for endocytosis. In this review we describe the evidence that NDP kinase function is essential for endocytosis and also elaborate on a mechanism for NDP kinase regulation of dynamin. Recently documented interactions between endocytosis and cell signaling have revealed new insights into potential mechanisms of cancer. In this context, we discuss the possible relevance of NDP kinase and dynamin interaction for tumor suppression.

KEY WORDS: Shibire; synaptic vesicle recycling; behavior; Awd; GEF; GAP.

INTRODUCTION

Nucleoside diphosphate (NDP) kinase is a conserved enzyme that produces nucleoside triphosphates from diphosphates by high-energy phosphate transfer (Parks and Agarwal, 1973). NDP kinase is required for a variety of biological processes including cell growth, differentiation, and tumor metastasis (Kimura *et al.*, 2000). Its role in these diverse processes is likely to be mediated by its influence on functions of diverse regulatory molecules. For instance, its proposed interactions with the GTPases Rad and Rac1 and in c-*MYC* transcription may contribute to its functions in cell motility and cell growth (Otsuki *et al.*, 2001; Postel *et al.*, 2000; Zhu *et al.*, 1999).

Recent studies have revealed a previously unknown role for NDP kinase in controlling endocytosis through regulation of dynamin, a GTPase necessary for endocytosis (Krishnan *et al.*, 2001). The mechanism by which NDP kinase regulates dynamin is unusual and may provide hints into general mechanisms that underlie diverse functions of NDP kinase protein family. With the recognition that endocytosis plays crucial roles in cell signaling and that cross talk between endocytosis and signaling converges on various pathological conditions, especially cancer (Di Fiore and De Camilli, 2001), the link between NDP kinase and dynamin may even prove to be significant for understanding NDP kinase function in tumor suppression.

In this review we first describe the role of dynamin in endocytosis and then elaborate on a model for NDP kinase regulation of dynamin and finally discuss the implication of this interaction in tumor metastasis.

ENDOCYTOSIS AND DYNAMIN FUNCTION

The process of endocytosis begins with vesicle-mediated entry of extracellular molecules and often culminates with delivery of these molecules to degradative compartments called lysosomes (Robinson *et al.*, 1996). Vesicles begin to form on the inner surface of the plasma membrane through local assembly of vesicle coat proteins such as clathrin around cytosolic tails of specific transmembrane proteins. Clathrin "coated pits" bud from the plasma membrane and are detached through concerted action of a set of fission proteins that include the GTPase dynamin (De Camilli *et al.*, 1995; Kirchhausen, 2000). After internalization, the vesicle uncoats, and, through a membrane fusion event, transfers cargo molecules into a cellular compartment called the endosome. Once in the endosome, the molecule can have different fates such as recycling back to the cell surface or trafficking to the lysosome for degradation (Gruenberg and Maxfield, 1995). Though

[1]Department of Molecular and Cellular Biology and ARL, Division of Neurobiology, University of Arizona, Tucson, Arizona.

[2]To whom correspondence should be addressed at Department of Molecular and Cellular Biology, Life Sciences South, Box 210106, University of Arizona, Tucson, Arizona 85721; e-mail: rnarayan@u.arizona.edu.

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endocytosis can occur by a variety of distinct mechanisms, the clathrin-mediated endocytic pathway has been recognized as an essential cellular process involved in nutrient uptake, antigen presentation, cell fate specification, regulation of activated growth factor receptors, and synaptic transmission (De Camilli and Takei, 1996; Dubois *et al.*, 2001; Futter *et al.*, 1996; Kramer *et al.*, 1991; Vieira *et al.*, 1996).

An essential role for dynamin in detaching clathrin coated vesicles from the plasma membrane is revealed by phenotypes associated with *Drosophila shibire* (*shi*^{ts}) mutations that have conditional defects in dynamin function (Chen *et al.*, 1991; Kosaka and Ikeda, 1983; Poodry and Edgar, 1979; van der Bliek and Meyerowitz, 1991). At a permissive temperature of 19°C, files with the *shi*^{tsl} mutation appear normal, but exposure to 27°C results in rapid behavioral paralysis. This behavioral paralysis is paralleled by a block in endocytosis at an advanced stage in vesicle formation, after the formation of invaginated pits (Fig. 1) (Kosaka and Ikeda, 1983; Ramaswami *et al.*, 1994). The necks of the invaginated pits trapped in *shi*^{tsl} mutant nerve termini have electron dense collars that probably include dynamin. Similar electron dense collars containing dynamin immunoreactivity are also present in mammalian synaptosomal membranes treated with GTPγS, a nonhydrolysable analog of GTP (Takei *et al.*, 1995). Addition of GTP to the membranes relieved GTPγS induced inhibition of endocytosis and permitted dynamin to separate from the neck of the vesicle; thus, GTP hydrolysis seems necessary for vesicle fission.

The conditional mutations in the *shi* gene map to the GTPase domain in dynamin (Fig. 2) (Grant *et al.*, 1998; van der Bliek and Meyerowitz, 1991). Analogous point mutations in the GTPase region of mammalian dynamin also lead to a block in endocytosis during the initial stages of clathrin assembly, as indicated by transferrin uptake studies (Herskovits *et al.*, 1993; van der Bliek *et al.*, 1993). These studies demonstrate a critical role for the GTPase domain in dynamin function. In addition, dynamin also contains several other distinct functional domains (Fig. 2): the plekstrin homology domain (PHD), that likely functions in phospholipid binding (Salim *et al.*, 1996; Shaw, 1996); the middle domain (MD), not well characterized but mutated in several lethal alleles of *shi* (Grant *et al.*, 1998); a proline rich domain (PRD) that mediates interaction with partner or substrate proteins such as amphiphysin (Shupliakov *et al.*, 1997); and, finally, a GTPase effector domain (GED) that may function as an intramolecular GTPase activating protein (GAP) to mediate dynamin assembly-stimulated GTP hydrolysis (Muhlberg *et al.*, 1997; Sever *et al.*, 1999).

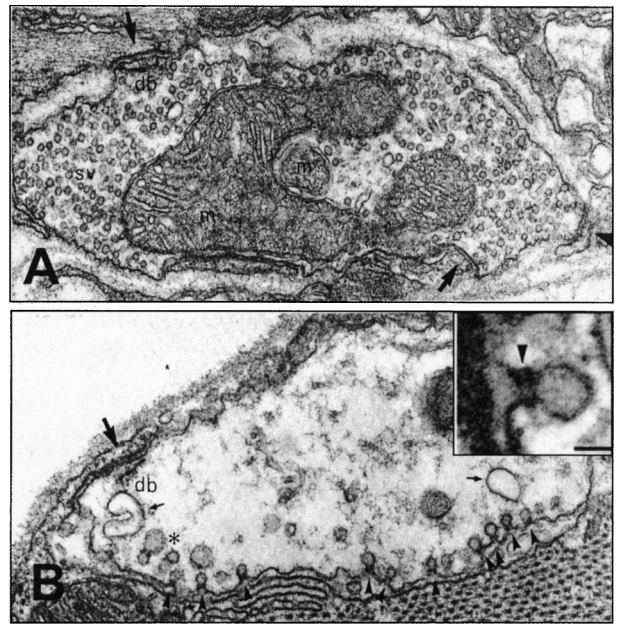

Fig. 1. Essential role for dynamin in endocytosis. EM cross section of *Drosophila* cervical synapses in a *shi* mutant (A) under permissive conditions (19°C) where the nerve terminal is wild type in morphology and is filled with synaptic vesicles. (B) Nerve terminal in a *shi* mutant after 8-min exposure to nonpermissive temperatures (29°C). Note the depletion of synaptic vesicles and a number of collared pits along the plasma membrane (arrowheads) in (B) compared to (A). Vesicle depletion and collared pits result from a block in vesicle internalization in *shi* mutant nerve terminal (Koenig and Ikeda, 1989; Kosaka and Ikeda, 1983). The inset in (B) shows an enlarged collared pit with electron dense structure (arrowhead) that probably includes dynamin. Reproduced with permission from the Society for Neuroscience.

Dynamin is an unusual GTPase in more than one way. Firstly, the presence of an intramolecular GAP activity in dynamin is unique among GTPases. Also, it exhibits a low affinity for GTP, with a K_M of about 30 mM, close to the resting cytosolic concentration of GTP; in addition, it shows high endogenous rates of GTP hydrolysis (Maeda *et al.*, 1992; Sever *et al.*, 2000; Shpetner and Vallee, 1992; Tuma *et al.*, 1993). These biochemical properties make dynamin function highly sensitive to the concentration of readily available GTP. In this context, the discovery that NDP kinase acts as an unconventional Guanine nucleotide exchange factor (GEF) for dynamin (Krishnan *et al.*, 2001) provides important novel insights into dynamin function and regulation.

NDP KINASE IS REQUIRED FOR DYNAMIN FUNCTION

The *Drosophila* NDP kinase is encoded by the *abnormal wing discs* (*awd*) locus (Biggs *et al.*, 1990; Rosengard

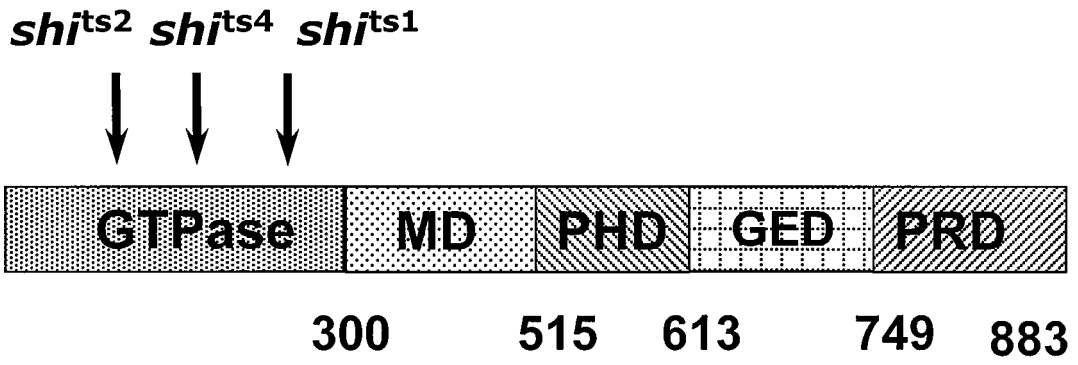

Fig. 2. Domain structure of dynamin. Dynamin has distinct functional domains that function in GTP binding and hydrolysis, the GTPase domain; phospholipid binding, the plekstrin homology domain (PHD); protein–protein interactions, the proline rich domain (PRD); a domain of unknown function, the middle domain (MD); and a domain involved in regulating assembly-stimulated GTP hydrolysis, the GTPase effector domain (GED). All the temperature sensitive mutations in *shi* map to the GTPase domain (Grant *et al.*, 1998; van der Bliek and Meyerowitz, 1991).

et al., 1989). Though NDP kinase activity in *Drosophila* has long been known to be essential for organismal survival (Xu *et al.*, 1996), precise cellular functions of NDP kinase remained elusive (Timmons and Shearn, 2000) until classical genetics revealed the unexpected connection between Awd/NDP kinase and dynamin in synaptic function (Krishnan *et al.*, 2001). Viable mutations in *awd*, such as *MSM95* that has about 10% of the normal NDP kinase activity, were isolated from a genetic screen designed to identify dynamin-interacting molecules. Viable, partial loss-of-function mutations in *awd* show strong synergism with temperature-sensitive *shi*^{ts} mutations. Thus, while *shi*^{ts2} mutant flies paralyze rapidly at 27°C (but not at all at 25°C), *shi*^{ts2}, *awd*^{MSM95} double mutants paralyze within 2 min at 22°C (Krishnan *et al.*, 2001).

Several additional lines of cell biological evidence clearly indicate that Awd activity in vivo is necessary for efficient dynamin function (Krishnan *et al.*, 2001). The presence of Awd/NDP kinase at synapses suggests that Awd is optimally positioned to regulate dynamin function at a cellular location where dynamin is critically required for its role in synaptic-vesicle endocytosis. Mutations in *awd* not only lower the temperature at which behavioral paralysis occur in *shi*^{ts} mutants but also lower the temperature at which *shi*^{ts} mutants show distinct cellular phenotypes of dynamin inhibition. Thus, characteristic blocks in synaptic transmission, and synaptic vesicle endocytosis are both seen at far lower temperatures in *shi*^{ts2}; *awd* double mutants than in *shi*^{ts} (Fig. 3). Strikingly, a specific allelic combination of *awd* (*awd*^{MSM95}/*awd*^{KrS6}) also exhibits *shi*^{ts} like phenotypes in an otherwise wild-type background. Thus, at 39°C, *awd*^{MSM95}/*awd*^{KrS6} mutants show behavioral paralysis, blocked synaptic transmission, and synaptic vesicle recycling defects characteristic of *shi* mutants (Fig. 3). Moreover, since dynamin levels are not altered in *awd* mutants (Krishnan *et al.*, 2001), these data affirm that Awd protein functions to positively regulate dynamin function at the synapse. An essential role for NDP kinase activity in this regulation is indicated by additional data. A mutant transgene *H119A3* (Xu *et al.*, 1996) that carries a mutation in a residue essential for NDP kinase activity is expressed at levels comparable to control wild-type transgenes. However, it cannot provide *awd* functions required for dynamin function whereas the wild type transgene can completely alleviate behavioral paralysis in *awd* mutants.

A possible mechanism by which NDP kinase regulates dynamin is suggested by observations showing that GTPases such as G proteins and microtubules may require local supplies of GTP generated by NDP kinase (Kimura and Johnson, 1983; Wieland and Jakobs, 1992; Zhu *et al.*, 1999). Thus, efficient dynamin function may depend on GTP provided by NDP kinase.

NDP KINASE IS A GEF FOR DYNAMIN

Dynamin is a GTPase with an unusually low affinity for GTP and, therefore, unlike other GTPases requires high concentrations (more than 30 μM) of cellular GTP for optimal function. In principle, this high concentration of GTP could be supplied either via a global action of NDP kinase in the cytosol, or by its action in the environment local to dynamin. Two lines of data support a local mechanism. First, evidence supporting a direct physical interaction between dynamin and NDP kinase (Baillat *et al.*, 2002) indicates that NDP kinase may be optimally positioned to provide a local supply of GTP for dynamin. Interaction between NDP kinase and dynamin, documented

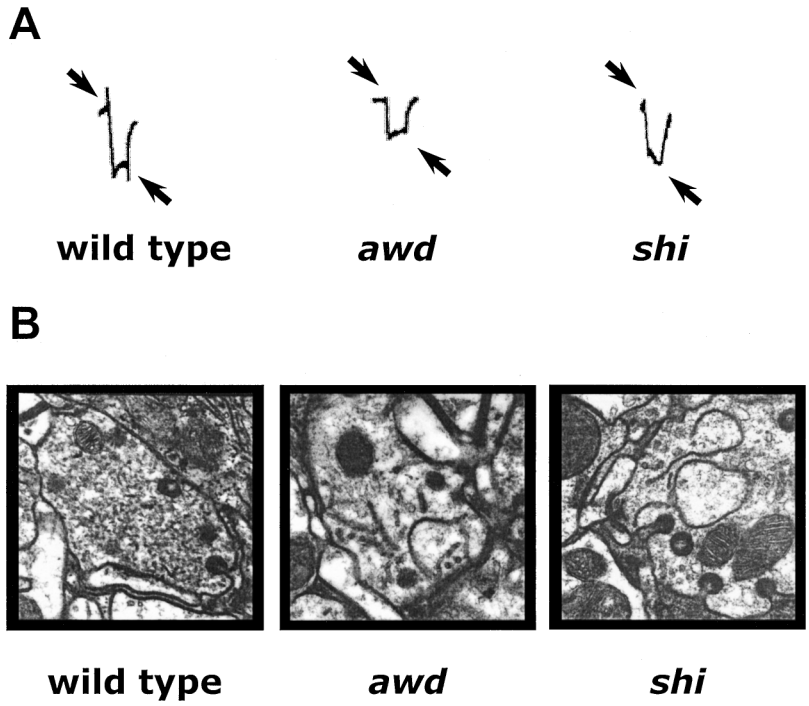

Fig. 3. Mutations in *awd* show *shi* like synaptic defects. Electroretinograms or ERGs from the adult eye (A) indicate that awd^{MSM95}/awd^{KrS6} mutants show synaptic failure at 39°C similar to shi^{CK2} mutants at 36°C. The spikes marked by the arrows represent synaptic responses of the optic lobe. Note the absence of these spikes in *awd* and *shi* mutants at their nonpermissive temperature while wild type shows synaptic response at 39°C (Krishnan *et al.*, 2001). (B) EMs of adult photoreceptor nerve terminals in awd^{MSM95}/awd^{KrS6} show arrest of membrane intermediates at 39°C similar to shi^{CK2} at 36°C. Note the presence of characteristic tubular recycling intermediates in *awd* and *shi* synapses whereas these structures are absent in wild type synapses at 39°C (Krishnan *et al.*, 2001). Reproduced with permission from Elsevier Press.

through immunoprecipitation and GST pull down experiments, occurs through the C-terminal proline-rich domain of dynamin. Moreover, the finding that Eps15, a protein required for clathrin-mediated endocytosis that also binds dynamin through the proline-rich domain, is part of the dynamin–NDP kinase containing complex indicates that NDP kinase is associated with a complex of proteins that play essential roles during endocytosis.

Second, genetic analyses in *Drosophila* argue that localization of NDP kinase to regions close to dynamin is essential for its normal function in endocytosis. Human NDP kinase transgenes can express substantial NDP kinase activity in *Drosophila* tissue (Xu *et al.*, 1996). However, *Drosophila awd* transgenes that provide substantially less NDP kinase activity than Human transgenes are far more effective at providing kinase activity required for Awd function in endocytosis (Xu *et al.*, 1996). The most economical interpretation (Xu *et al.*, 1996) of this observation is that NDP kinase activity must be targeted to a specific local context, such as the nerve terminal, to provide functions required for dynamin activity (Krishnan *et al.*, 2001). This targeting is probably achieved by sequence specific interactions that have diverged through evolution. Together with data showing a physical interaction between NDP kinase and dynamin in vitro, this in vivo requirement for NDP kinase targeting provides strong support for a model in which by locally supplying GTP, NDP kinase acts as an unconventional GEF for dynamin.

Further insight into the role of NDP kinase's GEF activity is provided by analysis of a NDP kinase insensitive mutant of dynamin. As GTPases must hydrolyze GTP and couple GTP hydrolysis to conformational change, it is predicted that dynamin mutations may exist in which these activities, downstream of GTP loading are defective. Such mutations should be relatively insensitive to reduced NDP kinase function. The first *shi* allele to be isolated, shi^{tsl}, appears to be such an allele (Chen *et al.*, 2002). Thus, while shi^{tsl} mutants paralyze at 27°C, which is identical to shi^{ts2} mutants, mutations in *awd* do not alter the temperature at which shi^{ts1} mutants paralyze. Consistent with a

post-GTP loading defect, *shi*^{ts1} mutant dynamin shows abnormal subcellular localization when compared to a large collection of NDP kinase sensitive *shi* alleles. These observations provide significant support for the notion that dynamin, in *shi*^{ts1} functions normally in NDP kinase dependent steps of endocytosis but is defective in a GEF independent step that leads to a block in endocytosis. The existence of such an allele also strongly argues against models indicating indirect effects of *awd* on vesicle endocytosis (Deitcher, 2001).

A MODEL FOR DYNAMIN FUNCTION

The unusual biochemical properties of the dynamin GTPase with its low affinity for GTP and high rates of GTP hydrolysis fits in nicely with a GEF activity regulating the critical GTP loading step of dynamin. The GEF activity mediated by NDP kinase should push dynamin into an active GTP bound form (Fig. 4). NDP kinase sensitive alleles of *shi*, like *shi*^{ts2}, are proposed to be defective in GTP affinity or GTP loading. The one NDP kinase insensitive allele *shi*^{ts1} may be defective in dynamin activities after GTP loading. For instance, it may be trapped in a metastable GTP-bound conformation that shows altered membrane binding upon exposure to high temperature. Thus, the interactions of NDP kinase and dynamin suggest a model for dynamin function where GTP loading by NDP kinase stabilizes the GTP bound form of dynamin (Fig. 4); subsequent GTP hydrolysis and/or a conformational change in dynamin (Marks *et al.*, 2001) that is independent of GTP availability comprise additional rate-limiting steps in the dynamin cycle.

ENDOCYTOSIS AND TUMOR SUPPRESSION

A role for NDP kinase as a GEF in regulating dynamin implicates NDP kinase function in endocytosis. While it is well known that endocytic processes play a role in downregulation of receptors involved in signaling pathways that lead to desensitization (Koenig and Edwardson, 1997), recent studies have indicated that endocytosis regulates signaling in multiple ways and interference with endocytic machinery leads to cell transformation and neoplasia (Di Fiore and De Camilli, 2001). The c-Cbl protein is an E3 ubiquitin-conjugating enzyme involved in endocytic sorting of growth factor receptors. When the function of c-Cbl is inhibited by mutations, it leads to prolonged receptor signaling and oncogenesis (Levkowitz *et al.*, 1998). This indicates that ubiquitination and regulation of growth factor endocytosis have tumor suppressive properties. Cross-talk between ubiquitination and endocytic pathways in tumor suppression is also demonstrated by the finding that TSG101, an E2 ubiquitin-conjugating enzyme, is involved in endocytosis and mutations that impair its function lead to defects in endosomal trafficking and results in a tumorigenic phenotype (Babst *et al.*, 2000).

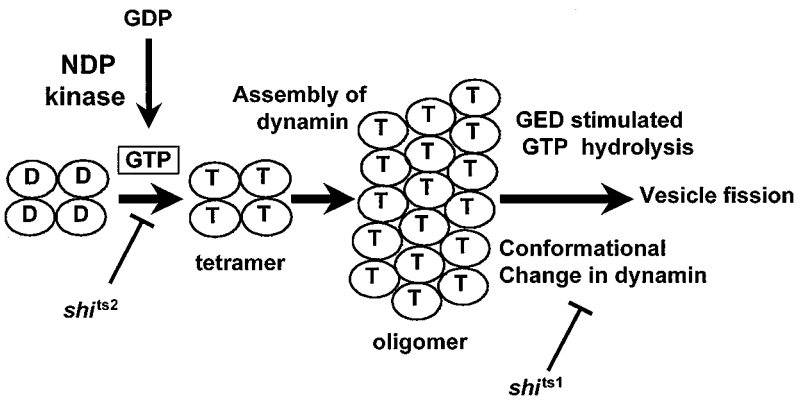

Fig. 4. Model for dynamin function. In this model dynamin exists as GDP bound tetramers and GTP loading by GEF activity of NDP kinase enables assembly of GTP bound dynamin into oligomers. Downstream of GTP loading, GTP hydrolysis of dynamin is stimulated by an intramolecular GAP activity encoded by the GED (Sever *et al.*, 1999) and a conformational change in dynamin (Marks *et al.*, 2001) occurs to complete vesicle fission. Though shown as a linear process, dynamin's role in endocytosis is likely to involve cycling between GDP and GTP bound states facilitated by NDP kinase activity and GAP activity in dynamin. The existence of NDP kinase sensitive (*shi*^{ts2}) (Krishnan *et al.*, 2001) and insensitive (*shi*^{ts1}) (Chen *et al.*, 2002) mutations in *shi* suggests that they affect different steps during this dynamin cycle; the probable functions affected in these mutations are indicated.

Also, a striking role for endosome membrane invagination in the attenuation of tyrosine kinase receptor (TKR) and epidermal growth factor receptor (EGFR) signaling clearly shows that endocytosis is involved in downregulation of signaling events (Lloyd *et al.*, 2002). Normally, invagination of the endosomal membrane removes the signaling domain from the cytoplasmic side resulting in signal attenuation. Mutations that reduce Hrs, an endosomal protein, not only result in defective budding of the endosome membrane but also lead to increased TKR signaling. These studies clearly show that endocytosis mediated signal attenuation has tumor suppressive properties and alterations in this control mechanism leads to neoplasia.

The suppressive effects of NDP kinase expression in murine and human *nm23*-deficient metastatic tumor lines clearly define a role for NDP kinase as a "metastasis suppressor" (Hartsough and Steeg, 2000; Leone *et al.*, 1991). In addition, stable transfection of *nm23H1* into murine melanoma cell lines and human breast carcinoma cells results in loss of migration in response to a variety of chemo attractants (Kantor *et al.*, 1993; MacDonald *et al.*, 1996). While biological functions for NDP kinase in tumor suppression are not well characterized and are independently discussed in this issue of *Journal of Bioenergetics and Biomembranes*, its role in endocytosis and evidence implicating endocytosis in tumor suppression may provide insight into the tumor suppressive properties of NDP kinase.

A positive effect of NDP kinase on dynamin-dependent endocytosis means that NDP kinase function is necessary for attenuation of signaling from growth factor receptors. Impairment of NDP kinase function in *nm23*-deficient tumors possibly results in prolonged signaling that triggers metastasis. Additionally, the evidence that NDP kinase and dynamin are associated with cellular components that mediate Ca^{2+} signaling events (Baillat *et al.*, 2002) suggests that cross-talk between endocytosis and signaling events plays a critical role in tumor progression.

CONCLUSIONS

In conclusion, this review describes how a genetic study to understand dynamin function in endocytosis revealed a surprising novel role for NDP kinase in acting as a GEF for dynamin as well as new insights into dynamin regulation. The role of NDP kinase in dynamin-dependent endocytosis and the exciting discoveries that implicate endocytosis in tumor suppression suggest an interesting possibility that the tumor suppressor properties of NDP kinase derive from dynamin regulation in endocytosis. Further studies aimed at regulation of dynamin function during endocytosis will go a long way in elucidating the multiple cellular functions of NDP kinase.

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