MECHANISM OF ACTIVATION OF NDR PROTEIN KINASE BY THE HMOB1 PROTEIN

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Samuel J. Bichsel
aus Hasle, Bern

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von:

Prof. Dr. Thomas Bickle, Prof. Dr. Patrick Matthias
Dr. Brian A. Hemmings

Basel, den 5.April 2005

Prof. Dr. Hans-Jakob Wirz
Dekan der Philosophisch-
Naturwissenschaftlichen
Fakultät

TABLE OF CONTENTS

1. Acknowledgements

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2. Summary

Serine/threonine kinases of the nuclear Dbf2-related (NDR) family are highly conserved throughout the eukaryotic world. Members of this kinase family are implicated in various aspects of the regulation of cell division and cell morphology. It has been shown that the function of several NDR kinases is dependent on proteins of the Mob (MPS one binder) family. MOB proteins are highly conserved throughout the eukaryotic world as well, which indicates the existence of a novel conserved signalling pathway.

The current work focuses on the mechanism of activation of human NDR kinase by the human MOB1 protein. hMOB1 directly activates NDR by binding to the N-terminal domain of NDR, thereby hMOB1 acts as a kinase activating subunit of NDR kinase. The binding induces the release of an autoinhibition caused by an autoinhibitory sequence (AIS) and leads to increased phosphorylation of NDR kinase on the two important regulatory phosphorylation sites Ser-281 and Thr-444.

3. General Introduction

3.1. Signal transduction

3.1.1. Overview

Cells in multicellular organisms communicate with each other to coordinate their growth and differentiation, and the biological processes of a cell have to be performed in a coordinated manner. Signalling cascades can be initiated extracellularly (binding of a ligand to a membrane receptor initiate a signalling cascade, Karin and Hunter, 1995) as well as intracellularly (e.g. mitotic checkpoints, Elledge, 1996). Signals have to be integrated to induce finally the proper physiological response, so signal transduction cascades consist in fact rather of networks than of simple linear pathways (Bhalla and Iyengar, 1999).

Classically, a signal transduction pathway is initiated by the binding of a ligand to its receptor in the cell membrane, the receptor transduces the signal into the cell to other signalling molecules, and finally, after transduction of the signal over several steps, the appropriate biological response is induced (Seger and Krebs, 1995).

The final targets of signal transduction cascades are the regulation of gene expression or the direct modulation of already existing enzymes or other cell components to induce a biological response (Keegan and Zamorano, 1998).

Signal transduction serves to respond to extracellular signals or to intracellular events and leads to the regulation of cell growth and cell division, cellular metabolism, cell morphology and cell differentiation (Abraham, 2002; Jones and Kazlauskas, 2001; Vaudry et al., 2002; Stachowiak et al, 2003; Fanto and McNeill, 2004; Knust, 2001).

3.1.2. Signalling molecules

There is a wide range of molecules that are involved in signal transduction cascades.

Receptor proteins serve to transduce a signal from outside the cell into the cell. The binding of the appropriate ligand (in some cases other stimuli) induces a conformational change of the receptor that results in transduction of the signal (Janeway et al, 1989). This is achieved by several mechanisms depending on the receptor type: activation of kinase activity by dimerisation/oligomerisation and autophosphorylation, recruiting of other molecules to the receptor (receptor tyrosine kinases, Taha and Klip, 1999, receptor serine/threonine kinases, Lin et al, 1992), association with other molecules (TNF receptors, Baker and Reddy, 1996), release of attached proteins (seven-transmembrane domain receptors, Casey and Gilman, 1988).

The most frequent signalling molecules are kinases which phosphorylate either tyrosine or serine/threonine residues. Tyrosine- and serine/threonine kinases can phosphorylate the next kinase in a signalling cascade or other proteins and alter thereby the structure and activity of these proteins. Tyrosine phosphatases and serine/threonine phosphatases are the counterparts of the kinases; they dephosphorylate proteins (Hunter, 1995).

A more detailed discussion of the importance of phosphorylation/dephosphorylation events and the structure of protein kinases will be given in the next sections.

Heterotrimeric G-proteins are the proteins associated with seven-transmembrane domain receptors. The Gα as well as the Gβγ subunits transduce signals. The Gα-subunit has GTPase activity. It its GTP-bound state it is turned on and interact with other proteins to transduce the signal, as soon as GTP is hydrolysed to GDP the Gα-subunit is turned off, this means it no longer interacts with the other molecule to transduce the signal. The Gα-subunit also interacts with proteins that modulate its

activity (GTPase activating proteins, guanine-nucleotide exchange factors) (Neer, 1995).

Monomeric GTPases are similar to the Gα-subunit of heterotrimeric G-proteins; they bind in the GTP-bound state to its effector molecules as well (Hall, 1992).

The hydrolysis of membrane constituents can generate second messengers. Phospholipases hydrolyse phospholipids. Phospholipase C hydrolyses phosphatidylinositol-3,4-bisphosphate and thereby generates the second messengers (second messengers are small diffusible molecules generated intracellularly in response to extracellular stimuli) diacylglycerol (which activates PKC) and inositoltrisphosphate (which induce a elevation of the intracellular Ca^{2+}- level). PLC's are regulated by heterotrimeric G-proteins (PLCβ's) or by receptor tyrosine kinases (PLCγ) (Kapeller and Cantley, 1994). PLD hydrolyses phosphatidylcholine to phosphatidic acid (PA) and choline. PLA2 converts PA to lysophosphatidic acid (LPA). PA and LPA can serve as second messengers (Liscovitch et al, 2000). Sphingomyelinases hydrolyse sphingolipids to ceramide and phosphatidylcholine. Ceramide serves as a second messenger (Ohanion and Ohanion, 2001).

Phosphatidyinositol kinases phosphorylate the inositol ring of phosphoinosits at the D3 (PI-3-kinase) D4 (PI4-kinase) or D5 (PI5-kinase) position. PI-4 and PI-5 kinase is required to generate PtdIns-4-5- P2, the substrate of PLC. The products of PI-3 kinase, Ptd-3-4-P2, Ptd-3,4,5-P3 act as second messengers (Kapeller and Cantley, 1994).

Several other kinases with similarity to PI-3 kinase are known, and some of them have, like PI-3 kinase, both lipid and protein kinase activity (Hunter, 1995). Phosphatidylinositol phosphatases are the counterparts of the phosphatidylinositol kinases (Myers et al, 1998).

Adaptor proteins have no enzymatic activity; they serve to bring other signalling molecules in close proximity to each other. The localisation of a signalling molecule is usually important for its activation (Pawson and Scott, 1997).

cAMP and cGMP are second messengers. The levels of them are regulated by adenyl/guanyl cyclases (regulated by G-protein coupled receptors, and the guanyl cyclases also by nitric oxide) and cAMP/cGMP phosphodiesterases (Conti and Jin, 1999; Schlossmann et al, 2003). These second messengers activate the protein kinases PKA and PKG, respectively.

3.2. Protein phosphorylation:

Proteins get phosphorylated by the transfer of the γ-phosphate group of adenosintrisphosphate to the hydroxylgroup of tyrosine, serine or threonine. This reaction is catalysed by two groups of enzymes, tyrosine kinases, which catalyse the phosphorylation of tyrosine residues, and serine/threonine kinases, which catalyse the phosphorylation of serine and threonine residues (Krebs, 1983). As an exception, there are also dual specific kinases known which can phosphorylate all three residues (Becker and Joost, 1999) Tyrosine and serine / threonine kinases show a high degree of similarity, they seem to have evolved by divergent evolution. Phosphorylation is a reversible reaction. Protein phosphatases catalyse the hydrolysis of a phosphate group from either tyrosine or serine/threonine residues (as a exception, also dual specific phosphatases exist.) (Tonks and Charbonneau, 1989; Wera and Hemmings, 1995). Tyrosine and serine/threonine phosphatases have a low degree of similarity in their primary structure; they seem to have evolved by convergent evolution.

The phosphorylation/dephosphorylation of an amino acid residue can alter the conformation and thereby the function of a protein. The phosphorylation state of a protein is first of all dependent on the activity of the kinases and phosphatases that are targeted against the phosphorylation sites of the protein.

3.3. Classification of protein kinases

The human genome contains 518 kinase gens. Protein kinases are classified by similarities of their catalytic domains in seven main groups. (Manning et al, 2002):

The AGC (containing PKA, PKG and PKC families) group, the CAMK (calcium/calmodulin-dependent protein kinase) group, the STE (homologs of yeast Sterile 7, Sterile 11 and Sterile 20 kinases) group, the CMGC (containing CDK, MAPK, GSK3 and CLK families) group, the CK1 (Casein kinase 1) group, the TKL (Tyrosine kinase like) group and the TK (Tyrosine kinase) group. Most kinases fall into one of these major groups. Closely related kinases form kinase families, like the NDR family, which consists of NDR1, NDR2, LATS1 and LATS2. Some kinases have little or no sequence similarity to the kinase superfamily. These kinases are atypical protein kinases, for example DNAPK.

3.4. Structure of protein kinases

The primary structures of the catalytic domains of all kinases show a high degree of similarities to each other. This indicates that almost all protein kinases have a common ancestor and was evolved by divergent evolution. Because of the similar primary structure, it is expected that the overall three-dimensional structures of all kinase catalytic domains are similar. By X-ray crystallographie, it was confirmed that the overall three-dimensional structures of the kinase catalytic domains are similar (Hanks and Hunter, 1995). The structure of the catalytic domain of PKA as a model for serine/threonine kinases is discussed below:

The catalytic domain of PKA is built of two lobes. The catalytic site lies in the cleft between these two lobes. The smaller, N-terminal lobe (containing subdomains I-IV) forms a five-stranded antiparallel beta-sheet. This lobe is responsible for the binding of Mg-ATP. The larger, C-terminal lobe (subdomains V-XI) consists mainly of alpha helices. This lobe is responsible for substrate binding and catalysis.

The catalytic domains of kinases can be further divided into twelve subdomains. The subdomains contain small regions with higher homology. The structural and functional features of the subdomains are the following:

Subdomain I:

Subdomain I contains a beta strand-turn-beta strand motif. The turn consists of the consensus motif: Gly-X-Gly-X-X-Gly-X-Val, named the glycine rich loop. The beta strands cover the alpha- and beta phosphates of ATP, while the glycine rich loop helps to anchor ATP to the protein.

Subdomain II:

Subdomain II consists of a beta sheet with a small alpha helix towards the end. The residue Lys-72 in the consensus motif, AXK, is conserved in all kinases. This subdomain is important for kinase activity by interacting with the alpha and beta phosphates of ATP.

Subdomain III:

Subdomain III consists of a large alpha helix. The residue Glu-91 is invariant, this residue helps to stabilise the interaction between Lys-72 and the alpha and beta phosphates of ATP.

Subdomain IV:

Subdomain IV is a beta beta strand and contains no invariant residues.

Subdomain V:

This subdomain consists of a hydrophobic beta strand in the small lobe and a small alpha helix in the large lobe connected by a chain between them. The chain contains the residues Glu-121, Val-123 and Glu-127, which help to anchor Mg-ATP. Met-120, Tyr-122, Val-123 contribute to a hydrophobic pocket that surround the adenine ring of ATP. Glu-127 is in PKA important for substrate recognition.

Subdomain VIA:

Subdomain VIA is a large hydrophobic alpha helix with no invariant residues.

Subdomain VIB:

Subdomain VIB consists of two small hydrophobic beta strands and a loop between them. The loop contains the invariant residues Asp-166 and Asn-171 within the consensus motif: D-X-K-X-X-N. This loop is known as the catalytic loop of the kinase. The residue Asp-166 is the catalytic base of the enzyme, the acceptor of the proton from the hydroxyl group of the serine or threonine from the substrate. Lys-168 is important for the neutralisation of the local negative charge of the gamma-phosphate (This residue is usually replaced by arginine or alanine in tyrosine kinases). Asn-171 assists in the stabilisation of the catalytic loop and chelates Mg^{2+}, while Glu-170 is important for the stabilisation of the binding of ATP and the substrate in PKA.

Subdomain VII:

This subdomain consists of a beta strand-loop-beta strand. It contains the consensus motif Asp-Phe-Gly (DFG). Asp-184 is important for the orientation of the gamma-phosphate of ATP by chelating the Mg^{2+} that bridges the beta and gamma phosphate of ATP.

Subdomain VIII:

Subdomain VIII is a chain, which faces the cleft between the large and small lobe. This chain contains the consensus motif Ala-Pro-Glu (APE). Glu-208 stabilises the large lobe. This subdomain is important for substrate recognition. In PKA, Leu-198, Cys-199, Pro-202 and Leu-205 contibute to a hydrophobic pocket that protects the side chain of the hydrophobic residue at position +1 in the substrate consensus sequence of PKA (RRXSY, Y is a hydrophobic amino acid). Subdomain VIII contains the activation (or T-) loop. Many kinases are activated by phosphorylation of threonine or serine in this loop (Thr-197 in PKA).

Subdomain IX:

Subdomain IX is a large alpha helix. This helix is important for the structure of the kinase as well as for substrate recognition. Invariant Asp-220, this residue helps to stabilise the catalytic loop in subdomain VIA.

Subdomain X:

This subdomain is a small alpha helix with no invariant residues.

Subdomain XI:

Subdomain XI is build of a helix-loop-helix motif. Nearly invariant is the residue Arg-280. 9 to 13 residues down of the invariant Arg is the consensus motif His-X-aromatic amino acid-hydrophobic amino acid.

3.5. Regulation of serine/threonine kinases

3.5.1. Phosphorylation/dephosphorylation

Generally, kinases are regulated by phosphorylation and dephosphorylation of certain threonine/serine and tyrosine residues. Phosphorylation alters the conformation of the kinases and thereby their activity. Phosphorylation can increase or decrease the kinase activity of a kinase. In many kinases, an activating phosphorylation site has been mapped to the T-loop. Regulatory phosphorylation sites have also been mapped at other sites in the catalytic domain and in the carboxy-terminal regulatory domain. (Russo et al, 1996; Yang et al, 2002)

Some sites can be constitutively phosphorylated; others change their phosphorylation state. In some kinases, sites that are phosphorylation sites in other kinases are replaced with acidic residues (aspartate, glutamate), which have a similar negative charge as phosphate groups (Johnson et al, 1996). . The phosphorylation state of a kinase is determined by the activity of kinases (upstream kinases or the kinase which autophosphosphorylates itself) and phosphatases.

3.5.2. Subcellular localisation

The in vitro substrate specificity of different kinases often overlaps. So the question rises how these kinases can elicit their different functions. An important aspect in this context is the localisation of a kinase.

In the cell, the activity is also determined by the localisation of a kinase. Kinases have to localise in close proximity to their upstream regulators, for example to receptors or other membrane associated proteins. The modulation of the kinase by this proteins can

then in turn, a part from the alteration of the activity, induce the translocation of the kinase to another subcellular compartment.

The exact localisation of kinases then determines (a part from the substrate specificity) which substrates the kinase can phosphorylate (Griffioen and Thevelein, 2002). Kinases often form complexes with their modulators and substrates.

3.5.3. Protein-protein interactions

Kinases are not only regulated by phosporylation, often they are regulated by interaction with regulatory subunits. The binding of another protein can induce a conformational change of the kinase and thereby activate or inactivate it. The expression levels of the regulatory subunit can in these cases regulate the kinase activity, or the concentration of a low molecular weight ligand, for example GTP or second messengers like Ca^{2+}, which bind to the regulatory protein and thereby alters the interactivity of it with the kinase.

3.5.4. Second messengers

Second messengers are small diffusible molecules that are generated intracellularly in response to extracellular stimuli. Second messengers regulate the activity of kinases.

Four main mechanisms are known:

-Inducing the dissociation of an inhibitory subunit from a kinase catalytic domain, e.g. PKA, cAMP as second messenger (McKnight, 1991).

-Second messenger dependent association of an activating subunit with the kinase catalytic domain, e.g. CaM kinases, Ca^{2+} as second messenger (Nairn, 1990).

-Direct binding of the second messenger to the kinase e.g. PKC, Ca^{2+} and diacylglycerol as second messsengers (Ron and Kazanietz, 1999).

-Second messenger induced delocalisation, e.g. localisation of PKB by Ptd Ins3,4-bisphosphate to the membrane (Meier and Hemmings, 1999).

Some soluble low molecular weight regulatory molecules are not produced in response to extracellular stimuli, e.g. AMP that signals the metabolic state of the cell (Hardie et al, 1998).

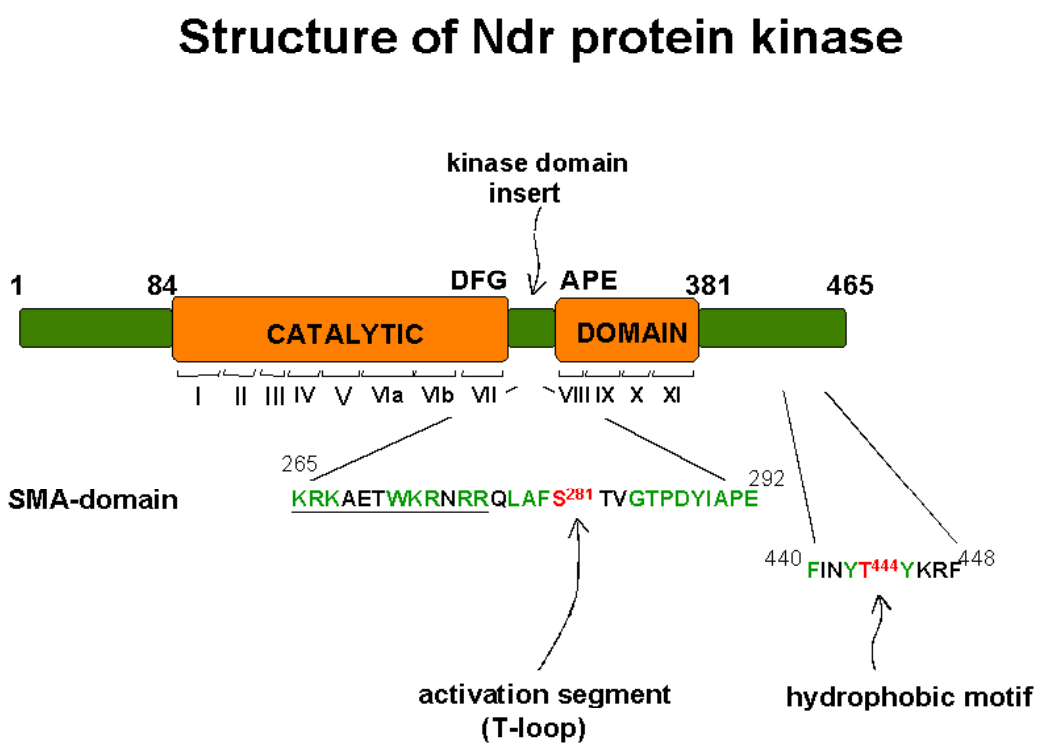
The diverse regulatory mechanisms (phosphorylation, regulation of the subcellular localisation, protein-protein interactions and second messengers) can act together to regulate a kinase.

4. NDR serine/threonine kinase

The NDR (nuclear-Dbf2-related) kinase was cloned from human fetal brain, D. melanogaster and C. elegans (Millward et al, 1995). NDR kinase belongs to a highly conserved family of kinases, a subclass of the AGC-family of kinases (Manning et al, 2002). The NDR family of kinases includes mammalian protein kinases NDR1 and NDR2, Drosophila melanogaster NDR/TRC, Caenorhabditis elegans Sax1, mammalian, D. melanogaster and C. elegans large tumour suppressor (LATS) kinases, Neurospora Cot1, Ustilago maydis UKC1, Saccharomyces cerevisiae Cbk1, Dbf2 and Dbf20, Schizosaccharomyces pombe Orb6 and Sid2 and a number of plant kinases (Tamaskovic et al, 2003).

4.1. Structure of human NDR kinase

The NDR serine/threonine kinase amino acid sequence shows all the twelve subdomains of the kinase catalytic domain (Hanks and Hunter, 1995). NDR kinases are most closely related to members of the AGC-group of serine/threonine kinases. However, the catalytic domain of NDR is interrupted by an insertion of about 30 amino acids between subdomain VII and VIII. This insertion into the catalytic domain is a special feature of NDR family kinases. The insertion is situated just in front of the phosphorylation site in the activation loop. The N-terminal regulatory domain is another characteristic region of NDR family kinases. This domain is highly conserved among NDR family kinases. It was shown that a region within this domain could bind directly to S100-Ca^{2+} binding proteins (Millward et al, 1998, Bhattacharya et al, 2003). Finally, the C-terminal extension contains a broadly conserved hydrophobic motif that is an important regulatory element within the AGC group of protein kinases (Yang et al, 1999).



4.2. Relatives of NDR

4.2.1. NDR is related to members of the AGC-group of kinases

NDR is related to members of the AGC-group, like PKA, PKB, PKG, PKCs, PRK, p70-^{S6} kinase, p90^{RSK}, SGK and PDK1. Typically, AGC kinases have in common the position of the important phosphorylation site in the activation loop and the one in the C-terminal regulatory domain, the hydrophobic motif site.

4.2.2. NDR family kinases

NDR is closely related to kinases found throughout the eukaryotic world (animals, plants and fungi). The NDR related kinases constitute a kinase family, the NDR family of kinases. These kinases all show a high conservation of at least a part of the N-

terminal domain, an interruption of the kinase catalytic domain between subdomains VII and VIII, and a conservation of the regions surrounding the regulatory phosphorylation sites. The members of the NDR family are more distantly related to other kinases such as the Ghengis Khan (GEK) kinases, DMPK and MRCK, the ROCK kinase and the budding yeast kinase RIM15. The function of NDR kinases has been conserved over long evolutionary distances, mainly involving various aspects of cell division and morphogenesis.

4.2.2.1 Saccharomyces cerevisiae Cbk1

Cbk1 is the closest relative of NDR in S. cerevisiae with an identity of the catalytic domain of 59%. Cbk1 mutants show large aggregates of unseparated cells, the cells are round instead of ellipsoidal, the apical growth is diminished and the budding pattern is random instead of bipolar. (Racki et al. 2000; Bidlingmaier et al, 2001). The localisation of Cbk1 is cell cycle dependent. During bud and mating projection formation, Cbk1 localises to sites of polarised growth and cell wall remodelling, such as the actively expanding cell cortex. During mitotic exit, Cbk1 moves to the bud neck and to the daughter cell nucleus. The localisation as well as the kinase activity of Cbk1 was shown to be dependent on several proteins that form a novel regulatory network. This network is, on the one hand, responsible for the daughter cell specific nuclear localisation of the transcription factor Ace2p, which controls the expression of genes important for the degradation of the septum, such as scw11 and cts1 encoding glucanase and chitinase. On the other hand, Cbk1 is important for polarised apical growth and mating projection formation, these functions are independent of the Ace2 transcription factor. The regulatory network that is controlling these two functions has been described as the RAM network (Regulation of Ace2p activity and cellular

morphogenesis). The RAM consists of Cbk1, Mob2, a small activator of NDR kinases, Tao3, a 270 kda protein of unknown molecular function, Hym1, a relative of human MO25, the Ste20 like kinase Kic1 and the leucine-rich repeat containing protein Sog2. All the components are required for Cbk1 kinase activity. The localization to the daughter nucleus of Cbk1-Mob2 depend on all other components of the RAM-network and all RAM components localize to sites of polarized growth and interact physically (Nelson et al, 2003). The components of the RAM network are conserved throughout the eukaryotic world.

4.2.2.2. Schizosaccharomyces pombe Orb6

Orb6 is the closest relative of NDR in S. pombe; its catalytic domain is 58 % identical to NDR. Orb6 was originally identified as one of 12 orb gens involved in cell morphogenesis (Verde et al, 1995). S. pombe cells switch in the early G2 phase to bipolar growth. The cells grow at the new tip that was formed by cell division (new end take off, NETO). The bipolar growth continues until mitosis. During these cell cycle transitions, profound changes in the actin cytoskeleton occur, involving formation of distinct actin patches at the growing tips in interphase and the actin ring during mitosis. Orb6 mutants loose growth polarity and become spherical, microtubules are disorganised and actin dots delocalised. Furthermore, Orb6 mutants enter mitosis earlier. On the other hand, an increase of the Orb6 levels delays mitosis and maintains cell polarity. Taken together, Orb6 is required to maintain cell polarity during interphase and to promote actin reorganisation after mitosis and during activation of bipolar growth.

Furthermore, Orb6 influences cell cycle progression by delaying the onset of mitosis. This delay is dependent on Orb6 kinase activity, and Orb6 affects the Cdc2 mitotic

kinase (Verde et al, 1999). The localisation of Orb6 protein changes during the cell cycle. During interphase, it is localised at the cell tips and during mitosis and cytokinesis at the region of the developing septum, which is consistent with a role of this kinase in polarised growth and cell division (Verde et al, 1999). Orb6 interact physically and genetically with the Furry-like protein Mor2/Cps12 and the S.pombe Mob2 protein (Hirata et al, 2002; Hou et al, 2003).

4.2.2.3. Saccharomyces cerevisiae Dbf2 and Dbf20

Dbf2 and Dbf20 are the second closest relatives of NDR kinase. Its catalytic domains are 40% identical to human NDR kinase. Temperature sensitive Dbf2 mutants arrest at restrictive temperature at the end of anaphase with large budded "dumbbell" morphology, and their mitotic spindles are elongated which is indicative of incomplete nuclear division (Johnston et al, 1990). The kinase activity of Dbf2 is cell cycle dependent, with a maximal activity in anaphase, when Dbf2 is localised to spindle pole bodies (SPB or yeast centrosomes) (Toyn and Johnston, 1994; Visintin and Amon, 2001). The localisation of Dbf2 changes later in mitosis, it moves to the bud neck prior to actin medial assembly, consistent with the role of Dbf2 in cytokinesis (Frenz et al, 2000). Dbf2 is part of a network described as the MEN (mitotic exit network). The MEN is important for the inactivation of mitotic cyclin dependent kinases and for the completion of cytokinesis. (Bardin and Amon, 2001). The mitotic exit network consists of the GTPase Tem1 and its exchange factor Lte1 (Shyrayama et al, 1994), the polo like kinase Cdc5 (Kitada et al, 1993), the Ste20 like kinase Cdc15 (Surana et al), Dbf2 and Dbf20 (Toyn and Johnston, 1994), the protein phosphatase Cdc14 (Visintin et al, 1998), and Mob1 (Komarnitsky et al, 1998). The MEN is inhibited by the Bub2 dependent spindle checkpoint (Fesquet et al, 1999). The MEN

gets activated after sister chromatid separation is completed. Tem1 binds to its exchange factor Lte1, probably enabled by the transport of the Tem1 carrying spindle pole body (SPB) to the daughter cell, where Tem1 is then in close proximity to Lte1. This leads to localization of Cdc15 to this SPB (Asakawa et al 2001), and activation of the NDR family kinase Dbf2 that is bound to Mob1 (Mah et al, 2001). Activation of the MEN finally leads to the release of the protein phosphatase Cdc14 from the nucleolus (Shou et al, 1999), which then triggers mitotic exit by dephosphorylation of Hct1 and Sic1 (Visintin et al, 1998). Dephosphorylated Hct1 binds to the anaphase promoting complex APC, an ubiquitin ligase. The APC-Hct1 complex leads to the destruction of mitotic cyclins, thereby inactivating Cdk (Zachariae et al, 1998). Sic1 is a Cdk inhibitor, and Swi5 is a transcription factor responsible for Sic1 expression. Sic1 as well as Swi5 are activated by dephosporylation (Visentin et al, 1998; Toyn et al, 1997; Verma et al, 1997). Furthermore, the polo like kinase Cdc5 acts in a complex way on MEN signalling: On the one hand, Cdc5 is required for Dbf2 activity, on the other hand, Cdc5 negatively regulate MEN signalling, acting upstream of Tem1 (Lee et al, 2001).

In addition to mitotic exit, MEN proteins are also directly involved in cytokinesis. It was shown that mob1 mutants displayed cytokinesis defects even under conditions where mitotic exit was not disturbed. In these mutants, the contractile ring at the bud neck was present, but the ring failed to contract and disassemble (Luca and Winey, 1998).

4.2.2.4. Schizosaccharomyces pombe Sid2

Sid2 is the S. pombe homologe of Dbf2. Its catalytic domain is 39% identical to that of human NDR kinase. Sid2 mutants are defective in septum formation during

cytokinesis (Balasubramanian et al, 1998; Sparks et al, 1999). Sid2 function in the SIN (Septation Initiation Network), a network that coordinates late mitotic events with cytokinesis (Mc Collum and Gould, 2001; Bardin and Amon, 2001). The SIN consists, a part from Sid2, of the small kinase activator protein Mob1 (Salimova et al, 2000), the GTPase Spg1 (Schmidt et al, 1997), the GTPase activating proteins (GAP) Cdc16-Byr4, which negatively regulate the SIN (Iwa and Song, 1998; Furge et al, 1998), the Polo like kinase Plo1 (Ohkura et al, 1995), the Ste20-like kinases Cdc7 (Fankhauser and Simanis, 1994) and Sid1 with its activating subunit Cdc14 (Fankhauser and Simanis, 1993; Guertin et al, 2000), the phosphatase Clp1 (Trautmann et al, 2001), and the scaffold proteins Cdc11 (Marks et al, 1992) and Sid4 (Balasubramanian et al, 1998). The SIN is required for actomyosin ring constriction and septum formation after chromosome separation is completed. The SIN proteins localize to the spindle pole body, where Sid2-gets activated when sister chromatide separation is completed. The activated Sid2-Mob1 complex translocates to the actomyosin ring prior to cell separation (Sparks et al, 1999).

4.2.2.5. Fungal kinases Cot1/Ukc1/Tb3

Neurospora crassa Cot1, Ustilago maydis Ukc1 and Colleotrichum trifolii TB3 have an identity of their catalytic domains of about 50% to human NDR, and the regulatory domains are well conserved. N. crassa, U. maydis and C. trifolli are filamentous fungi that grow by apical extensions of their hyphal tips. The hyphae branch and form a new growing tip after elongation. In Cot1 mutants, the elongation of the hyphaes is prevented and the hyphaes branch more frequent, therefore Cot1 mutants grow slower and form compact colonies (Yarden et al, 1992). C. trifolii TB3 can complement N.

crassa Cot1 mutants (Buhr et al, 1996). Ukc1 mutants are not able to generate aerial filaments during mating (Duerrenberger and Kronstad, 1999).

4.2.2.6. Plant NDR family kinases

Several NDR family kinases were identified in plant genomes, but their function was not yet described.

4.2.2.6. Protozoan protein kinases

In Protozoans, NDR is conserved in Trypanosoma species, but not in other clinical relevant species like Plasmodia. NDR related kinases were also discovered in Euplotes (Tan et al, 2001).

4.2.2.7. LATS kinases in mammalians and Drosophila

Drosophila and human LATS (Large tumour suppressor) kinase posses a catalytic domain which is about 55% identical to human NDR, but the regulatory important regions of NDR (phosphorylation sites, N-terminal domain) are less conserved than in the other NDR family kinases. The N-terminal domain of NDR is longer than that of NDR, and it contains glutamine and proline rich (Drosophila LATS) or proline rich (human LATS) stretches. In these respects, the LATS kinases are similar to Cbk1 and the fungal kinases Cot1, Ukc1 and TB3.

Drosophila LATS/Wts mutants were isolated in a mosaic screen designated to discover overproliferation mutations that are lethal at early developmental stages (Justice et al, 1995; Xu et al, 1995). Therefore, mitotic recombination in somatic cells was induced and thereby chimaeric animals with clones of mutated cells were

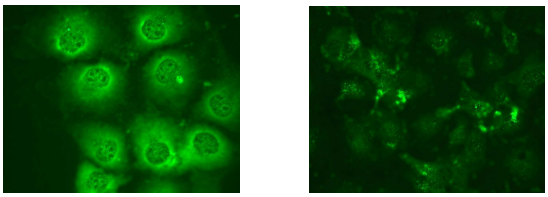
obtained. Clones of homozygous Lats mutant cells overproliferate and produce spectacular outgrowths. Clones of Lats mutants are round or spherical shaped, so the cells of the imaginal discs have not divided oriented as in the case of wild type imaginal discs. Furthermore, Lats mutant cells have an altered cell shape. Taken together, loss of LATS/Wts function is lethal during development and causes in genetic mosaic clones overproliferation, disoriented cell divisions and a change of the cell morphology. In humans exist two close relatives of Drosophila LATS/Wts, hLATS1 and hLATS2/Kpm. LATS1 kinase affects cell proliferation. Transduction of LATS1 into many tumor cell lines induces an inhibition of cell proliferation, and ectopic expression of LATS1 specifically reduces the cyclin A and cyclin B protein levels and thereby induces a reduction of CDK1 activity, which leads to growth arrest in the G2/M phase. It can also promote apoptosis by the induction of the Bax protein level (Xia et al, 2002, Yang et al, 2001). Furthermore, LATS1 was reported to affect cytokinesis by the inhibition of LIMK1 (Yang et al, 2004). Human LATS1 is phosphorylated in a cell cycle dependent manner; it is phosporylated at late prophase and throughout metaphase and gets dephosphorylated at anaphase. It was reported that LATS1 interacts directly with CDC2 in early mitosis when LATS1 is phosphorylated, and thereby inhibits CDC2 activity directly (Tao et al, 1999). LATS1 localises to the centrosomes during interphase, translocates to the mitotic spindle during metaphase and anaphase and to the midbody in telophase (Nishiyama et al, 1999). Mouse LATS knock out mice displayed impaired mammary gland development, infertility, growth retardation and the development of soft tissue sarcomas and ovarian stromal cell tumors (St. John et al, 1999). Taken together, LATS1 acts as a tumor suppressor by negatively regulating cell proliferation and by modulating cell survival. Human LATS2 kinase, an isoform of LATS1 kinase, negativly regulates cell growth by

inducing G2/M arrest and apoptosis as well (Kamikubo et al, 2003). LATS2 kinase localises to centrosomes and is a phosphorylation target of Aurora-A kinase (Toji et al, 2004).

4.3. Localisation of NDR kinase

Human NDR1, which was overexpressed in COS-1 cells, was detected in the nucleus. (Millward et al, 1995). By further localisation studies it was shown that NDR is not primarily localised to the nucleus but rather also to membranes and the cytosol, and endogenous NDR phosphorylated on threonine 444 was localised to membranes and the cytosol (Bichsel, unpublished, see figure; Hergovich et al, manuscript in preparation).

A) B)



Localisation of Thr-444 phosphorylated, endogenous NDR in COS-1 cells. A) Localisation with anti-P-Thr-444 antibody. B) Control, competition with antigen (T444-phosphopeptide).

4.4. Regulation of NDR kinase by phosphorylation:

While there is no physiological upstream signal known to stimulate NDR kinase activity, NDR1 and NDR2 is potently activated in vivo by treatment with the PP2A-inhibitor okadaic acid. Okadaic acid treatment of COS-1 cells leads to phosphorylation of the activation segment site Ser-281/Ser-282 and the hydrophobic motif site Thr-

444/Thr-442 (Millward et al, 1999; Stegert et al, 2004). These sites are also important regulatory phosphorylation sites in other kinases of the AGC group (Parker and Parkinson, 2001). It was shown for the prototypical AGC kinase PKA that he activation segment residue aligns in its phosphorylated form the catalytic site of this kinase, thereby generating an active kinase conformation (Knighton et al, 1991). The structure of PKBβ confirmed this role of the activation segment residue and defined the function for the hydrophobic motif. The hydrophobic motif site, upon phosphorylation, undergoes a series of interactions with αB- and αC-helices of the catalytic domain, thereby promoting disorder to order transition of this part of the molecule. This transitions leads to restructering of the activation segment and reconfiguration of the kinase bilobal structure (Yang et al, 2002).

The activation segment site is an autophosphorylation site in vivo. Kinase inactive NDR becomes not phosphorylated at that position, the kinase activity of NDR is required for the phosphorylation of the activation segment (Tamaskovic et al, 2003). In most other AGC kinases, including PKB, $p70^{S6K}$, SGK, $p90^{RSK}$ and PKC isoforms, however, the activation segment site is phosphorylated by phosphoinositide dependent kinase PDK1 (Belham et al, 1999). Some AGC kinases become phosphorylated in absence of PDK1 (PKA, PKCδ, AMPK, MSK1 and PRK2) (Williams et al, 2000; Balendran et al, 2000). NDR is not phosphorylated by PDK1, neither in vivo nor in vitro (Millward et al, 1999). This may be due to the fact that the NDR sequence surrounding the activation segment site is different in the P+1 and P+2 position to the highly conserved PDK1 consensus target site (Ser/Thr-Phe-Cys-Gly-Thr-Xaa-Asp/Glu-Tyr-Xaa-Ala-Pro-Glu, where Ser/Thr is the phosphoacceptor site and Xaa stands for a hydrophobic residue). Taken together, NDR is not targeted by PDK1 but it

becomes efficiently autophosphorylated at the activation segment residue both in vivo and in vitro.

Kinase inactive NDR become phosporylated on the hydrophobic motif phosphorylation site to the same extend as wild type NDR in vivo. Thus, NDR becomes phosphorylated by an upstream kinase. The hydrophobic motif phosphorylation site is conserved amongst almost all AGC group kinases. The hydrophobic motif site of NDR is phosphorylated by the Ste20 like kinase MST3 (Stegert et al, manuscript in preparation). The S. cerevisiae NDR relative Dbf2 was shown to become phosphorylated by the Ste20 like kinase Cdc15 (Mah et al, 2001), and Kic1, a close relative of MST3, is part of the Cbk1 involving RAM-network (Nelson et al, 2003). Furthermore, in S.pombe, the Ste20 like kinase Sid1 is placed upstream of the NDR relative Sid2 (Guertin et al, 2000), and Pak1 genetically interacts with Orb6 (Verde et al, 1998). Taken together, Ste20 like kinases are likely to accomplish the role to phosphorylate the hydrophobic motif of NDR kinases. The hydrophobic motifs of other AGC group kinases are likely to be phosphorylated by different kinases. For PKB, it was shown that DNA-dependent kinase (DNA-PK) acts as one of the hydrophobic motif upstream kinases (Feng et al, 2004).

Ca^{2+} is required for NDR kinase activation in vivo, depletion of Ca^{2+} by the intracellular Ca^{2+}-Chelator BAPTA-AM leads to reduced phosphorylation of the activation segment- as well as of the hydrophobic motif phosphorylation sites. Ca^{2+}-bound S100B binds to an amphiphilic helical region of the N-terminal domain of NDR, and S100B binding increases NDR autophosphorylation as well as transphosphorylation activity in vitro. (Millward et al, 1998; Tamaskovic et al, 2003; Stegert et al, 2004). NDR kinase is activated in a number of S100B overexpressing melanoma cell lines (Millward et al, 1998). Given the facts that S100B is bound to

OA-stimulated and unstimulated NDR and that an increase of the Ca^{2+} level by treatment with calcium ionophores induces only a slight activation, Ca^{2+}/S100B may be necessary, but not sufficient, for NDR activation to occur in vivo.

4.5. Expression of NDR1 and NDR2 kinase

In mammalians, two NDR isoforms exist, NDR1 and NDR2. There seem to be no obvious differences regarding the posttranslational regulation of these two kinases, but they differ in their tissue specific distribution. By *Taq*Man real-time PCR analysis, the highest expression levels of m*Ndr1* were observed in spleen, lung, thymus, brain, and fat tissue, whereas m*Ndr2* expression was found mainly in the large and small intestine, as well as in the stomach and testis. Assuming similar PCR efficiency for both reactions, m*Ndr2* appears to be the predominantly expressed isoform in mice. These data suggest tissue-specific functions of NDR1 and NDR2 in mammals (Stegert et al, 2004).

4.6. Substrates of NDR kinases

To date, no physiological substrate, neither of NDR kinase nor of one of the other NDR family kinases, has been discovered. NDR is able to efficiently phosphorylate a synthetic peptide with the sequence KKRNRRLSVA (Millward et al, 1998).

5. Introduction MOB proteins

New Tasks for the MOB

unpublished

Samuel J. Bichsel, Alexander Hergovich, Rastislav Tamaskovic, and Brian A. Hemmings*

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

*Corresponding author

Brian A. Hemmings

Friedrich Miescher Institute for Biomedical Research

Maulbeerstrasse 66

CH-4058 Basel

Switzerland

Phone: +41 61 697 4872

FAX: +41 61 697 39 76

e-mail:Brian.Hemmings@fmi.ch

Abstract:

Serine/threonine kinases of the nuclear Dbf2-related (NDR) family are highly conserved throughout the eukaryotic world. Members of this kinase family are implicated in various aspects of the regulation of cell division and cell morphology. It has been shown that the function of several NDR kinases is dependent on proteins of the Mob (MPS one binder) family. Mob proteins are highly conserved as well, and in humans they constitute a group of seven related proteins. In the last couple of years, it turned out that Mob proteins act as kinase activating subunits for NDR family kinases, similar to the role cyclins are playing in activating cyclin dependent kinases (Cdks).

Article outline:

1. **Introduction**
2. **The MOB protein family**
3. **Functions of MOB proteins in yeast**

3.1. S. cerevisiae

3.2. S. pombe

4. **Function of MOB in other organisms**
5. **Mechanism of activation of NDR kinase by MOB proteins**
6. **Concluding remarks**

1. Introduction

NDR (nuclear Dbf2-related) kinases constitute a family within the AGC group of serine/threonine kinases (1). They all contain the 12 subdomains of the kinase catalytic domain as described by Hanks and Hunter (2). However, the NDR kinase structure possesses unique features: First, their catalytic domain is interrupted by an insertion of about 30-60 amino acids between subdomains VII-VIII. Second, the N-terminal regulatory domain is unique and highly conserved among the NDR family of kinases. NDR family kinases are implicated in the regulation of cell division and cell morphology (3).

In 1998, Komarnitzsky et al have shown that S. cerevisiae Mob1 interacts physically and genetically with Dbf2, a yeast NDR family kinase (4). S. cerevisiae Mob1 was shown before to be an essential gene required for the completion of mitosis and maintenance of ploidy (5). MOB proteins are highly conserved during evolution. In the last couple of years, it has been revealed that MOB proteins play an important role for the function of NDR family kinases. MOB proteins influence the function of NDR kinases in Saccharomyces cerevisiae, Schizosaccharomyces pombe and Homo sapiens. In this article, we review the current knowledge about the important role MOB proteins are playing in concert with kinases of the NDR family.

2. The MOB protein family

MOB proteins constitute a protein family of highly conserved proteins; they are found in vertebrates, insects, worms, fungi, plants and protozoans with an identity of the amino acid sequences of at least 47 % (Fig.1). In humans, the MOB proteins diverged in at least six isoforms: hMOB1A and B, hMOB2, hMOB3A, B, and C. Furthermore, another protein termed “phocein” is also closely related to the MOB proteins (Fig.2). This divergence is conserved in other mammalian organisms, for example mice, which also possess MOB1, MOB2, MOB3 and phocein proteins, with an identity of the amino acid sequences between 92 and 100% to the respective human isoforms. Despite the high diversity of the MOB protein family in mammalians, there was no function known for these proteins until recently. MOB proteins also contain no known functional domains. The part of the amino acid sequence, which aligns well amongst all MOB family members (amino acids 29-213 of hMOB1A), is referred to as the Mob1_Phocein domain (Pfam accession nr. PF03637).

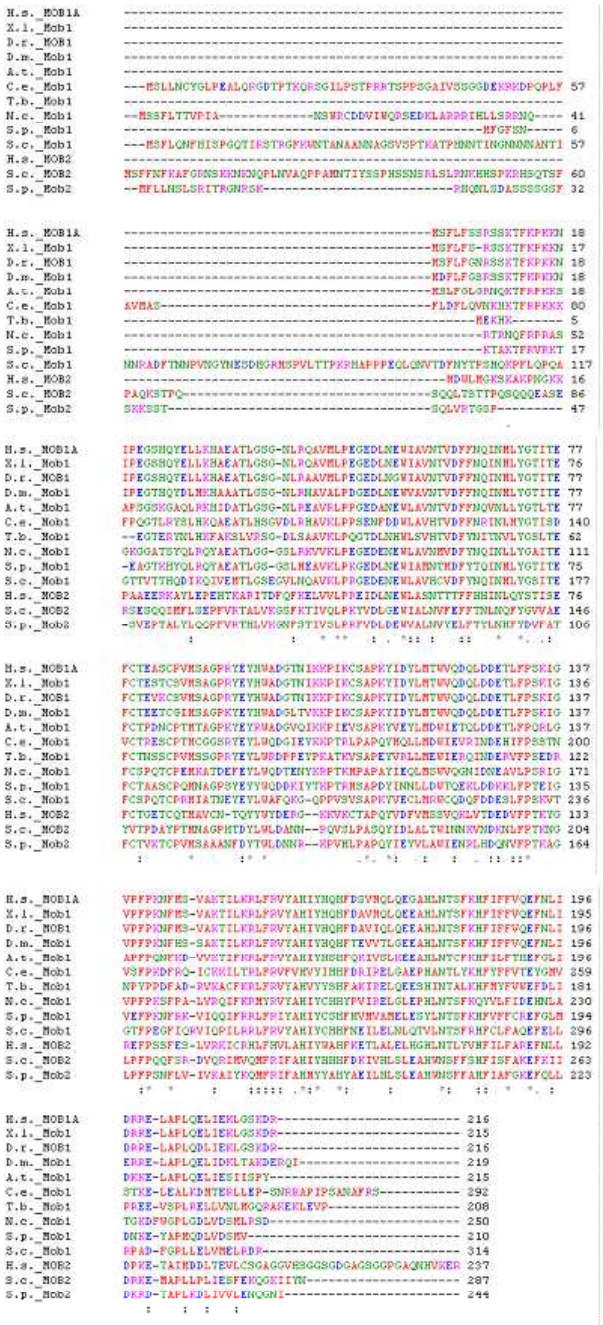


Fig.1: Alignement of the amino acid sequences of MOB proteins.

Fig. 2: Alignement of the amino acid sequences of human MOB proteins

3. Functions of MOB proteins in yeast:

3.1. *S. cerevisiae*

S. cerevisiae MOB1 was originally identified as an essential yeast gene (6) and in a yeast two-hybrid screen as an interactor with MPS1, a dual specificity protein kinase required for spindle pole body duplication and for the mitotic checkpoint (7). However, further studies have shown that Mob1 is responsible for different functions than Mps1 (5). Mob1 acts in the mitotic exit network (MEN) and in the control of cytokinesis (8). The mitotic exit network consists of the GTPase Tem1 and its exchange factor Lte1 (9), the polo like kinase Cdc5 (10), the Ste20 like kinase Cdc15 (11), the Ndr-family kinases Dbf2 and Dbf20 (12), the protein phosphatase Cdc14 (13) and Mob1 (4). The MEN is inhibited by the Bub2 dependent spindle checkpoint (14). The MEN gets activated after sister chromatid separation is completed. Tem1 binds to its exchange factor Lte1, probably enabled by the transport of the Tem1 carrying spindle pole body (SPB) to the daughter cell, where Tem1 is then in close proximity to Lte1 (15). This leads to localization of Cdc15 to this SPB (16), and activation of the NDR family kinase Dbf2 that is bound to Mob1 (17). Activation of the MEN finally leads to the release of the protein phosphatase Cdc14 from the nucleolus (18), which then triggers mitotic exit by dephosphorylation of Hct1 and Sic1 (19). Dephosphorylated Hct1 binds to the anaphase promoting complex APC, an ubiquitin ligase. The APC-Hct1 complex leads to the destruction of mitotic cyclins, thereby inactivating Cdk (20). Sic1 is a Cdk inhibitor, and Swi5 is a transcription factor responsible for Sic1 expression. Sic1 as well as Swi5 are activated by dephosporylation (19,21,22). Furthermore, the polo like kinase Cdc5 acts in a

complex way on MEN signalling: On the one hand, Cdc5 is required for Dbf2 activity, on the other hand, Cdc5 negatively regulate MEN signalling, acting upstream of Tem1 (23).

In addition to mitotic exit, MEN proteins are also directly involved in cytokinesis. It was shown that mob1 mutants displayed cytokinesis defects even under conditions where mitotic exit was not disturbed. In these mutants, the contractile ring at the bud neck was present, but the ring failed to contract and disassemble (8). The MEN components Dbf2 and Mob1 are localized to the spindle pole body (SPB) during anaphase but subsequently move to the bud neck, consistent with their role in cytokinesis (24). The localization of Dbf2 and Mob1 is dependent on the activity of the protein phosphatase Cdc14, which is high when mitotic exit is completed (8,25). Taken together, Mob1 and the NDR family kinase Dbf2 are important components of signalling networks required for exit from mitosis and the completion of cytokinesis. Mob1 is required for the kinase activity of Dbf2 and binds directly to Dbf2 (4,23). In vitro, Mob1 and the Ste20-like kinase Cdc15 are sufficient to promote the phosphorylation of the activation segment site Ser-374 and the hydrophobic motif site Thr-544. Cdc15 alone does not efficiently phosphorylate Dbf2 (17).

S. cerevisiae Mob2 is 90% identical to Mob1. Mob2 interacts with the NDR family kinase Cbk1 (26), the closest relative of human NDR kinase. Cbk1 has two different functions: it is important for the maintenance of polarized cell growth and is required for cell separation (27). Cell separation, the degradation of the septum after cytokinesis, requires the transcription of genes controlled by the Ace2 transcription factor (28. Cbk1 kinase activity is required for Ace2-dependent transcription. Mob2 is required for the kinase activity as well as for daughter-cell specific localisation of Cbk1 (29,30). The control of polarized growth, on the other hand, is Ace2-

independent. During polarized growth, Cbk1 and Mob2 localise interdependently to the bud cortex, while they localize to the bud neck and the daughter nucleus late in mitosis. Cbk1 kinase activity is cell cycle dependent, the activity is high in G1, low after release from G1, high during budding and maximal late in the cell cycle when the cells are large budded, consistent with the role of Mob2-Cbk1 in mating projection formation and cell separation. The Cbk1-Mob2 network is connected to the MEN network, since signalling from the MEN is required for nuclear localization of Cbk1-Mob2. (30). Other components of the network, which regulates Ace2 dependent transcription, and the maintenance of polarized growth were discovered. The RAM network (Regulation of Ace2p activity and cellular morphogenesis) consists of Cbk1, Mob2, Tao3, a 270 kda protein of unknown molecular function, Hym1, a relative of human MO25, the Ste20 like kinase Kic1 and the leucine-rich repeat containing protein Sog2. All the components are required for Cbk1 kinase activity. The localization to the daughter nucleus of Cbk1-Mob2 depends on all other components of the RAM-network and all RAM components localize to sites of polarized growth and interact physically (31). The components of the RAM network are conserved throughout the eukaryotic world.

3.2. *S. pombe:*

In S. pombe, the components of the MEN and RAM signalling networks are well conserved. S. pombe Mob1 is required for the initiation of medial ring constriction and septation. Mob1 localizes to spindle pole bodies throughout mitosis and to the cell division site later in mitosis. It interacts with the NDR family kinase Sid2, a close relative of S. cerevisiae Dbf2. Sid2-Mob function in the SIN (Septation Initiation Network), a network that coordinates late mitotic events with cytokinesis (32,33). The SIN consists, a part from Sid2 and Mob1, of the GTPase Spg1 (34), the GTPase

activating proteins (GAP) Cdc16-Byr4, which negatively regulate the SIN (35,36), the Polo like kinase Plo1 (37), the Ste20-like kinases Cdc7 (38) and Sid1 with its activating subunit Cdc14 (39, 40), the phosphatase Clp1 (41), and the scaffold proteins Cdc11 (42) and Sid4 (43). The SIN is required for actomyosin ring constriction and septum formation after chromosome separation is completed. The SIN proteins localize to the spindle pole body, where Sid2 gets activated when sister chromatide separation is completed. The activated Sid2-Mob1 complex translocates to the actomyosin ring prior to cell separation (44). For a detailed review of SIN and MEN signalling, see reviews (45-47). Mob1 is essential for Sid2 kinase activity and localization and binds directly to the N-terminal domain of Sid2 (48), a region that is highly conserved amongst NDR-family members.

The serine/threonine kinase Orb6 is the closest relative of human NDR and budding yeast Cbk1 in S. pombe. Orb6 is important for maintaining the cell polarity and for the coordination of cell morphogenesis with the cell cycle. Reduction of the Orb6 level leads to loss of polarized cell shape and to mitotic advance, while overexpression of Orb6 delays mitosis by affecting the p^{34}cdc2 mitotic kinase. The delay is dependent on Orb6 kinase activity. Orb6 localizes to the cell tips during interphase and to the division site during mitosis (49). Orb6 genetically interacts with the Tao3/Pag1 relative Mor2/Cps12. Mutation of Mor2/Cps12 results in lost of cell polarity, the same phenotype as the Orb6 knock-down, and a G2 delay, as observed by Orb6 overexpression (50). The methyltransferase Skb1, which is implicated in cell cycle control, the control of the coordination of cell cycle progression with morphological changes and in hyperosmotic stress response, interacts with Orb6 (51). Skb1 as well as Orb6 genetically interact with the S. pombe p21-activated kinase Pak1/Shk1 (49,51). Skb1 affects the localisation of Orb6 (51). The NDR family kinase Orb6 interacts

genetically and physically with Mob2. The phenotypes of Mob2 mutants and Mob2 overexpression are identical to the ones described for Orb6 (52). Mob2 binds directly to the N-terminal domain of Orb6 (48), a domain that is highly conserved in all NDR family kinases.

Taken together, Mob proteins in budding and fission yeast are components of important signalling networks, which are involved in the coordination of the cell cycle with cell morphology. Mob proteins interact directly with NDR-family kinases and they act as activating subunits of these kinases, similar to the role cyclins play in regulating cyclin dependent kinases.

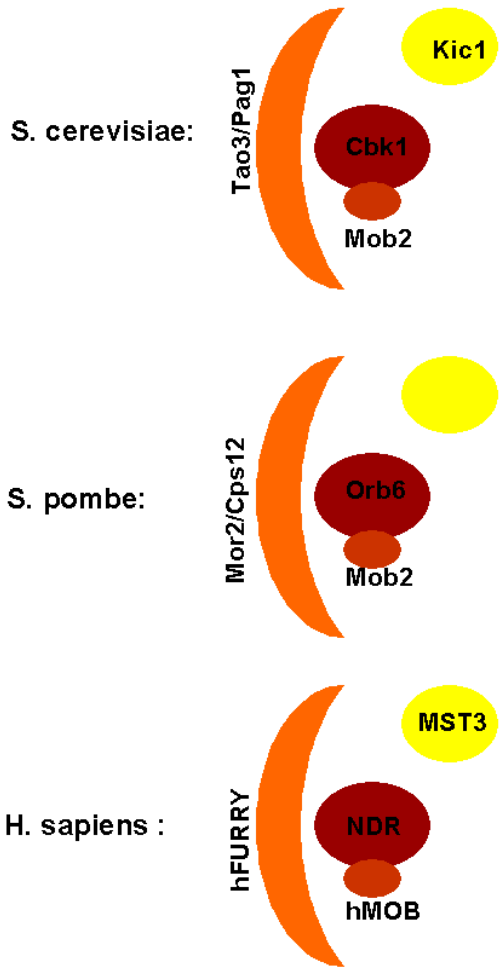


Fig.3: Conserved components of the RAM, Orb6 and NDR pathway in S. cerevisiae, S. pombe and H. sapiens. Cbk1/Orb6/NDR kinase binds to Mob proteins. This interaction is important for kinase activation. A Ste20 like kinase (Kic1/MST3) is supposed to be responsible for the phosphorylation of the hydrophobic motif site. The Tao3/Mor2/hFURRY proteins act most likely as scaffolding proteins.

4. Role of Mob in other organisms:

Proteins related to the components of the MEN, SIN and RAM networks are well conserved throughout the eukaryotic world. It is therefore likely that similar networks exist in other organisms. In human cells, NDR kinase physically interacts with MOB proteins. MOB proteins function as kinase activating subunits of human NDR kinase (53, 54). To date, nothing is published about a role for MOB proteins in cell cycle regulation and cell morphology in mammalians, insects and nematodes. But preliminary results in our lab point to a role for the MOB regulated kinase NDR in the control of the cell cycle and cell division: NDR activity and phosphorylation of its hydrophobic motif site Thr-444 is tightly cell cycle regulated. Activity and Thr-444 phosphorylation increases at the G1/S boundary and persists until mitosis and cytokinesis. Mouse embryonic fibroblasts derived from NDR1 knockout mice display severe proliferation defects, including delayed entry into S-phase accompanied by reduced expression of cyclin A, and decelerated mitosis. Elimination of NDR2 in mouse embryonic fibroblasts from NDR1 knockout mice leads to an exacerbation of the proliferation blocks, extensive cell death and to accumulation of polyploid and multinucleated cells, which is due to cytokinesis defects. (R. Tamaskovic et al, manuscript in preparation.). Human Mob1 was found to be localized to centrosomes (55). These data indicate that mammalian MOB proteins, by regulating NDR kinase, may have a similar role as the yeast MOB proteins in the regulation of the cell cycle and cell division.

The protein kinases LATS (large tumor suppressor) 1 and LATS2 are members of the NDR family of kinases as well; they localize to the mitotic apparatus and the centrosomes, respectively, and are involved in the regulation of the cell cycle and

apoptosis (56-60). Furthermore, LATS1 localizes to the actomyosin contractile ring during cytokinesis, and colocalizes with and binds to LIM kinase 1. LATS1 knockdown affects cytokinesis (61). But to date, there is no genetic or physical interaction between LATS and Mob proteins reported. On the other hand, Drosophila Lats kinase was shown to be in a pathway with Hippo (62-64), a Ste20 like kinase related to human MST2 and MST1. MST kinases are related to the MEN, SIN and RAM kinases Cdc15, Sid1 and Kic1. But confusingly, it was shown that hippo phosphorylates a part of the Drosophila Lats kinase that is not conserved in any other NDR family kinase (62).

5. Mechanism of activation of NDR kinase by MOB proteins.

NDR kinases belong to the AGC group of serine/threonine kinases. Human NDR1 and NDR2 are activated by phosphorylation of the activation segment site, Ser-281/282, and the hydrophobic motif site in the C-terminal domain, Thr-444/442 (66,67). The activation segment site is autophosphorylated, while the hydrophobic motif site is phosphorylated by a not yet identified upstream kinase (68). The signals that activate NDR kinase are not yet known. Treatment of cells with the protein phosphatase inhibitor PP2A induces activation of NDR kinase (66,67). Activation is in vivo dependent on Ca^{2+}, and the Ca^{2+}-binding protein S100B binds to a sequence in the N-terminal domain and activates NDR kinase in vitro (68,69).

It is shown that the human Mob proteins MOB1A, MOB1B and MOB2 interact with NDR kinase 1 and 2. The interaction of MOB1A with NDR is dependent on okadaic acid induced modification of MOB1A (54) MOB1A and MOB2 stimulate NDR kinase activity. (53, 54). MOB1A bind to the N-terminal domain of NDR, the SMA (S100 and MOB association) domain (54). The SMA domain is highly conserved among NDR family members which interact with MOB, and the S. pombe NDR kinases Orb6 and Sid2 interact with Mob proteins through their N-terminal domain as well (48) Highly conserved residues within the N-terminal domain are essential for NDR activity and interaction with MOB (54). A 30-60 amino acid insert between subdomains VII and VIII interrupts the kinase domain of all NDR family kinases. These insert act autoinhibitory, since the mutation of this AIS (auto inhibitory sequence) mutation leads to kinase activation in vitro. NDR with mutated AIS has a kinase activity similar to wild type NDR stimulated by MOB1A, and it cannot be significantly further stimulated by addition of MOB1A. These indicate that MOB1A

acts by inducing a conformational change that leads to the release of the autoinhibition. Furthermore, the AIS mutant has, apart from the increased kinase activity, an increased phosphorylation of Thr-444 in vivo (54). The release of the autoinhibition therefore facilitated phosphorylation by the Thr-444 upstream kinase. Interestingly, it is known for the S. cerevisiae NDR family kinase Dbf2 that binding of Mob1 facilitates phosphorylation by the Ste20 like kinase Cdc15 (17).

NDR kinase phosphorylated on the hydrophopic motif site Thr-444 is localised mainly to the cytoplasm, and hMOB1A and hMOB1B is found mainly in the cytoplasma as well, while hMOB2 localises to the cytoplasm and nucleus. All of these three hMOB isoforms co-localise with NDR in the cytoplasma and at certain sites at the plama membrane. Nuclear targeted NDR1 recruits hMOB1 to the nucleus. The co-localisation is dependent on the ability of NDR to bind to hMOB1, since a NDR form mutated on a residue that is important for MOB binding is important for co-localisation. Membrane targetting of NDR activates NDR kinase activity constitutively, and overexpression of hMOB1 further stimulates NDR kinase activity. Membrane targeting of hMOB1 leads to recruitment of NDR kinase to the plasma membrane and rapid stimulation of NDR kinase activity and phosphorylation on Ser-281 and Thr-444. These data suggest an *in vivo* model for NDR activation through rapid recruitment to the plasma membrane by hMOB proteins (Hergovich et al, manuscript in preparation).

The three dimensional structure of hMob1A has been solved by X-ray cristallography (70) and the one of the closely related Xenopus MOB1 by nuclear magnetic resonance (71). The core structure of these MOB proteins consists of a four-helix bundle that is stabilized by a bound zinc atom. The N-terminal helix of this bundle is solvent exposed and forms together with adjacent secondary structure elements an

evolutionary conserved surface. This surface is negatively charged and may interact with basic regions of NDR. Interestingly, S. cerevisiae Mob1 mutations that affect the biological function of Mob1 are localized on this surface, either affecting its charge or structure. For example, the acidic residue Glu-151 of S. cerevisiae Mob1 is changed in mutants that are unable to interact with Dbf2, and the corresponding conserved residue Glu-50 is situated on the negatively charged surface in hMOB1 (70,71).

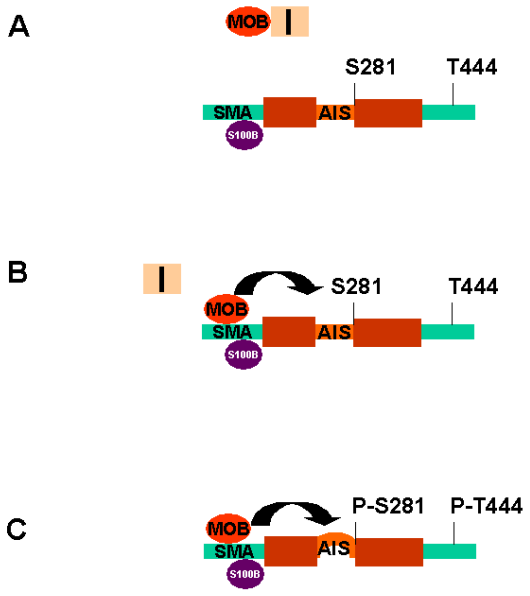


Fig.4. Model of NDR activation. A) Inactive state: Ser-281 and Thr-444 dephosphorylated by PP2A, insert in the catalytic domain prevents Ser-281 phosphorylation, MOB is perhaps bound to another protein X and, therefore, cannot bind to NDR. B) OA-induced modification of MOB enables MOB to interact with the N-terminal domain of NDR. C) MOB binding induces conformational change of NDR, thereby releasing the autoinhibitory effect of the insert. This increases autophosphorylation and the kinase activity of NDR. Phosphorylation of T444 by an upstream kinase leads to a fully activated kinase.

6. Concluding remarks

Mob proteins act as kinase activating subunits for kinases of the NDR family. They are involved in pathways that are important for the coordination of cell division with the cell cycle as well as for the regulation of cell polarity. Many questions concerning the role of a possible MEN- or RAM- like network in mammalian cells remain to be answered. Further investigation of these networks in mammalian systems is required for a better understanding of mammalian cellular biology. This could open new possibilities for drug development. NDR kinases and interacting proteins may be involved in tumor development: NDR1 mRNA is upregulated in highly necrotic and progressive ductal carcinoma in situ of the breast (DCIS) (72), and NDR kinase activity is elevated in several melanoma cell lines (69). NDR2 mRNA is upregulated in the highly metastatic non-small cell lung cancer cell line NCH-H460 (73). The mRNA encoding human Mob2 is upregulated in hepatocellular carcinomas (53,74). These findings suggest a role for NDR protein kinase in the regulation of cancer cell morphology and migration.

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6. Aim of the study

The human serine/threonine kinases NDR1 and NDR2 are members of the AGC group of kinases. Human NDR kinases belong to a kinase family that is highly conserved throughout the eukaryotic world. Members of this kinase family are implicated in the regulation of cell morphology and the cell cycle (Tamaskovich et al, 2003). In S. cerevisiae, signalling networks (RAM, MEN), implicating the NDR kinases Cbk1 and Dbf2, were discovered (Nelson et al, 2003, Bardin and Amon, 2001). The components of these networks are highly conserved proteins as well. The aim of the work presented herein was to further elucidate the activation mechanism of NDR kinase. Based on the high conservation of the components of the RAM and MEN networks, we investigated the effect of hMOB1, a conserved protein whose yeast relatives are part of the RAM and MEN signalling networks, on NDR kinase activity. Thereby we developed a new model for the activation mechanism of NDR kinases by MOB proteins, which act as kinase activating subunits. Furthermore, we further refined the phosphorylation mechanism of NDR, showing that NDR is phosphorylated on the activation segment site by autophosphorylation and on the hydrophobic motif site by an upstream kinase, and that both phosphorylations are Ca^{2+}- dependent.

7. Results
7.1 Mechanism of Activation of NDR kinase by the hMOB1 protein

Mechanism of activation of nuclear Dbf2-related (NDR) kinase by the hMOB1 protein

Samuel J. Bichsel, Rastislav Tamaskovic, Mario R. Stegert, and Brian A. Hemmings*

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

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*Corresponding author

Brian A. Hemmings

Friedrich Miescher Institute for Biomedical Research

Maulbeerstrasse 66

CH-4058 Basel

Switzerland

Phone: +41 61 697 4872

FAX: +41 61 697 39 76

e-mail:Brian.Hemmings@fmi.ch

Running title: Activation of NDR kinase by hMOB1

Summary

NDR (nuclear Dbf2-related) kinase belongs to a family of kinases that is highly conserved throughout the eukaryotic world. We showed previously that NDR is regulated by phosphorylation and the Ca^{2+} binding protein S100B. The budding yeast relatives of *Homo sapiens* NDR, Cbk1 and Dbf2, were shown to interact with Mob2 (Mps one binder 2) and Mob1, respectively. This interaction is required for the activity and biological function of these kinases. In this present study, we show that hMOB1, the closest relative of yeast Mob1 and Mob2, stimulates NDR kinase activity and interacts with NDR both *in vivo* and *in vitro*. Point mutations of highly conserved residues within the N-terminal domain of NDR reduced NDR kinase activity as well as hMOB1 binding. A special feature of NDR kinases is an insert within the catalytic domain between subdomains VII and VIII. A sequence within this insert shows a high basic amino acid content in all kinases of the NDR family known to interact with MOB proteins. We show that this sequence is autoinhibitory, and our data indicate that binding of hMOB1 to the N-terminal domain of NDR induces the release of this autoinhibition.

Introduction

NDR kinase[1] belongs to a highly conserved family of kinases, a subclass of the AGC (protein kinase A, G and C) group of kinases (1,2). The NDR family consists of the mammalian protein kinases NDR1 and NDR2, *Drosophila melanogaster* NDR, *Caenorhabditis elegans* SAX1, mammalian, *D. melanogaster* and *C. elegans* large tumour suppressor (LATS) kinases, *Neurospora crassa* COT1, *Ustilago maydis* UKC1, *Saccharomyces cerevisiae* Cbk1, Dbf2 and Dbf20, *Schizosaccharomyces pombe* Orb6 and Sid2, and several plant kinases (1,2). These kinases share a high sequence conservation and some possess conserved functions, mainly involving regulation of cell morphology and the cell cycle (2-15).

The kinase domain sequence of NDR is related to that of other members of the AGC group of kinases, e.g. PKA, PKG, PKCs, PRK, $p70^{S6K}$, $p90^{RSK}$ and PDK1 (1). NDR contains all 12 subdomains of the kinase catalytic domain as described by Hanks and Hunter (16). However, the catalytic domains of all members of the NDR family are interrupted by an insert of 30 – 60 amino acids between subdomains VII and VIII. This inserted sequence is not well conserved but is always rich in the basic amino acids arginine and lysine. The catalytic domain insert has been shown to act as a non-consensus nuclear localisation signal in the case of NDR1: NDR1 localises predominantly to the nucleus in COS1 cells, whereas mutant NDR1 with a deletion in the insert is localised to the cytosol (17). A further special feature of NDR-family kinases is an highly conserved N-terminal domain. In the case of NDR1, this domain consists of 81 amino acids and encompasses a region predicted to form an amphiphilic α-helix that binds to the EF-hand Ca^{2+} binding protein S100B (18). Finally, the C-terminal extension of

NDR kinase contains a broadly conserved hydrophobic motif phosphorylation site that is an important regulatory site within the AGC group of protein kinases (19). NDR kinase is efficiently (20 to 100-fold) activated upon treatment of cells with the PP2A inhibitor okadaic acid (OA). OA treatment induces phosphorylation of the activation segment site Ser-281 and the hydrophobic motif site Thr-444 (20). We have shown that Ser-281 is autophosphorylated, whereas Thr-444 is targeted by an as yet unidentified upstream kinase. Both sites are crucial for NDR activity *in vivo* and *in vitro*. NDR activation is Ca^{2+} dependent, as shown by treatment of COS1 cells with the Ca^{2+} chelator BAPTA-AM, which abolishes NDR activation. It has been shown that the EF-hand Ca^{2+} binding protein S100B binds to the N-terminal domain of NDR *in vivo* and *in vitro* and that Ca^{2+}/S100B activates NDR *in vitro*. S100B induces increased autophosphorylation on Ser-281. During investigations of the mechanism of S100B-induced autophosphorylation, a third autophosphorylation site, Thr-74 in the N-terminal domain, was discovered (21). This site is also crucial for NDR activation, since its mutation to alanine affected NDR activity *in vivo*.

The results of several recent studies indicate a novel, conserved signalling pathway involving NDR kinase family members. It has been shown in *D. melanogaster* that NDR genetically interacts with FURRY, a 300-kDa protein of unknown function (22). In *S. cerevisiae*, the FURRY relative Tao3/Pag1 lies on the Cbk1 pathway. Furthermore, Tao3/Pag1 and Cbk1 interact physically and their localisation is interdependent (23). In *S. pombe*, the FURRY-like protein Mor2/Cps12 interacts physically with Orb6, the *S. pombe* NDR orthologue (24). The FURRY-like proteins are conserved in mammals and, thus, it is likely that other proteins interacting genetically and/or physically with S. cerevisiae Cbk1 or

Dbf2 also play a role in the NDR kinase family pathway in higher eukaryotes. Most of these proteins are fairly well conserved throughout evolution.

S. cerevisiae Mob1 is a member of the mitotic exit network (MEN) (25, 26). Dbf2 associates with Mob1, and Mob1 is required for phosphorylation and activation of Dbf2 (27). *S. cerevisiae* Mob2, a close relative of Mob1, is a member of the Cbk1 pathway. Mob2 is required for the biological function of Cbk1 in mother/daughter separation after cytokinesis and maintenance of polarised cell growth. Mob2 associates physically with Cbk1, and Cbk1 kinase activity is dependent on Mob2. Furthermore, Mob2 and Cbk1 show interdependent localisation (28, 29). Similarly, *S. pombe* Mob2 interacts physically with the protein kinase Orb6 and is required for Orb6 function in the coordination of cell polarity with the cell cycle (30). Multicellular organisms possess highly conserved MOB proteins. hMOB1α shares a sequence identity/similarity of 50/65% with *S. cerevisiae* Mob2 and of 57/78% with *S. pombe* Mob1. The human MOB protein family consists of two almost identical proteins, hMOB1α and hMOB1β, (Gi8922671 and 27735029) sharing a sequence identity/similarity of 95/97%, a more distantly related protein, hMOB2 (Gi 38091156) that is 41/60% identical/similar to hMOB1α, three other related proteins, hMOB3 α, β and γ, (Gi18677731, 41350330 and 3809115) with an identity/similarity of about 50/73% to hMOB1α, and the weakly similar protein phocein (Gi41349451) that is 24/45% identical/similar to hMOB1α. Since the nomenclature of MOB proteins in the databases is rather confusing, we use the above terminology based on homology as also proposed recently by Stravridi et al. (31). To date, no functional domains have been identified in MOB proteins and the hMOB proteins have no known functions. It has been shown that the MOB

relative phocein interacts with the PP2A regulatory subunit striatin and with proteins involved in vesicular traffic (32,33).

Here, we characterise the interaction of hMOB1α, the closest relative of yeast Mob1 and Mob2, with human NDR kinase. We show that hMOB1 binding is dependent on the N-terminal domain of NDR and that hMOB1 stimulates NDR kinase activity both *in vivo* and *in vitro*. Furthermore, we show that a basic sequence within the insert in the catalytic domain of NDR has an autoinhibitory function and that hMOB1 may stimulate NDR activity by releasing the autoinhibitory effect of this sequence.

Experimental procedures

Cell culture – COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were transfected at the subconfluent stage with Fugene-6 transfection reagent (Roche) according to the manufacturer's instructions. In some experiments, the cells were treated for 60 min with 1μM OA in 0.1% *N,N*-dimethylformamide (DMF) or 50 μM BAPTA-AM in 0.1% DMSO 48 h after transfection.

Plasmids – Mammalian expression vector pCMV5 encoding HA-tagged NDR1 was described previously (21). pCMV5-hMOB1α was constructed by PCR using the Image clone 4854541 (BG 754693) as template and the primers 5`GGG GTA CCA CCA TGG AAC AGA AAC TCA TCT CTG AAG AGG ATC TGA GCT TCC TCT TCA GCA GCC GCT C3' and 5'GCT CTA GAC ATT TAT CTG TCT TTT GAT CCA AGT TTC TCT ATT AAT TCT TGA AGA GG3`, and subcloned into the KpnI and the XbaI sites of the vector. pGex2T-hMOB1 was constructed by PCR using the primers 5'CGG GAT CCA GCT TCC TCT TCA GCA GCC GCT C3' and 5'CCG CTC GAG CAT TTA TCT GTC TTT TGA TCC AAG TTT CTC TAT TAA TTC TTG AAG AGG3' and subcloned into the BamH1 and XhoI sites of the vector. For the bacterial production of NDR protein kinase, NDR2 was fused to a capsid-stabilising protein of lambdoid phage 21 (SHP)[2]. The cloning details and vector maps are available upon request. pCMV5 HA-NDR1 and pSHP-NDR2 point mutations were generated from wild-type vectors using the QuickChange site mutagenesis protocol (Stratagene) and

appropriate primers (primer sequences upon request). Sequences of all plasmids were confirmed by DNA sequencing.

Antibodies – Anti-Ser-281P and anti-Thr-444P antibodies were as described previously (21). Anti-Thr-74P rabbit polyclonal antiserum was raised against the synthetic peptide AHARKET(PO4)EFLRLK. The 12CA5 (HA) and the 9E10 (myc) monoclonal antibody hybridoma supernatants were used for detection of HA-NDR and myc-hMOB1. Anti-GST-NDR polyclonal antibody was as described previously (17).

Western blotting – To detect HA-NDR, SHP-NDR and myc-hMOB1, samples were resolved by 10% or 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) containing 5% skimmed milk powder and were then probed overnight at 4°C with anti-GST NDR rabbit polyclonal antibody, 12CA5 (HA) monoclonal antibody supernatant, 9E10 anti-myc monoclonal antibody supernatant, anti–Thr-444P, anti-Ser-281P or anti Thr-74P. Bound antibodies were detected with horseradish peroxidase-linked secondary antibodies or, for detection of myc-hMOB1 in HA-immunoprecipitations, with HRP-conjugated Protein A/G, and ECL.

Bacterial expression of human-GST-fused hMOB1 and -SHP-fused NDR2 – XL-1 blue *E. coli* was transformed with the pGEX-2T-hMOB1 plasmid. Mid-logarithmic phase cells were induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at 20°C. Bacteria were disrupted using a

French press in the presence of 1 mg/ml lysozyme and the fusion proteins were purified on glutathione-agarose. SHP-NDR2 wild-type and mutant plasmids were transformed into XL-1 blue E. coli and the protein produced as described for GST-hMOB1 and purified on Ni-NTA Sepharose.

HA-NDR kinase assay – Transfected COS-1 cells were washed once with ice-cold PBS and harvested in 1 ml ice-cold PBS containing 1 mM Na_3VO_4 and 20 mM β-glycerolphosphate before lysis in 500 µl of IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 20 mM β-glycerolphosphate, 1 µM microcystin-LR, 50 mM NaF, 0.5 mM PMSF, 4 µM leupeptin, and 1 mM benzamidine. Lysates were centrifuged at 20000 g for 20 min and duplicate aliquots (250 µg) of supernatant were precleared with protein A-Sepharose for 60 min and mixed subsequently for 3 h at 4°C with 12CA5 antibody prebound to protein A-Sepharose. The beads were then washed twice with IP buffer, once for 10 min with IP buffer containing 1 M NaCl, again for 10 min with IP buffer, and finally twice with 20 mM Tris-HCl pH 7.5 containing 4 µM leupeptin and 1 mM benzamidine. Thereafter, beads were resuspended in 30 µl buffer containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 µM [γ-^{32}P]ATP (~1,000 cpm/pmol), 1 µM cAMP-dependent protein kinase inhibitor peptide, 4 µM leupeptin, 1 mM benzamidine, 1 µM microcystin-LR and 1 mM NDR1 substrate peptide (KKRNRRLSVA). After 60 min incubation at 30°C, the reactions were processed as described previously (21).

SHP-NDR kinase assay – 1 µg purified recombinant SHP-NDR wild type and mutants (without further treatment or pre-autophosphorylated in the presence of

10 μM GST-hMOB1 or GST) were assayed in a 30 μl reaction mixture containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 μM [γ-^{32}P]ATP (~1,000 cpm/pmol) and 1 mM NDR1 substrate peptide (KKRNRRLSVA). After incubation at 30°C, reactions were processed and kinase activity determined as described for the HA-NDR kinase assay.

Immunoprecipitations – COS-1 cells transfected with HA-NDR wild type or mutants and myc-hMOB1 were harvested as described above. Cell lysate protein (0.5 mg) was precleared with protein A or G Sepharose and mixed subsequently for 3 h at 4°C with 12CA5 antibody prebound to protein A Sepharose or with 9E10-antibody prebound to protein G Sepharose. The beads were then washed twice with IP buffer, once with IP buffer containing 1M NaCl, once again with IP buffer, and finally twice with 20 mM Tris-HCl pH 7.5 containing 4 μM leupeptin and 1 mM benzamidine. Samples were resolved by 12% SDS-PAGE and myc-hMOB1 and HA-NDR detected by western blotting.

GST pull down – 25 μg aliquots of GST or GST-hMOB1 were incubated with gluthathione Sepharose for 2 h at 4°C. The beads were washed three times with TBS and then 5 μg aliquots of SHP-NDR wild type or mutants were added and incubated for 3 h at 4°C. The beads were washed five times with TBS, resuspended in 30 μl 1xSDS sample buffer and the samples resolved by 12% SDS-PAGE. NDR bound to GST-hMOB1 was detected by western blotting.

Results

hMOB1 activates NDR in COS1 cells as well as in vitro – Previous work by other groups has shown in yeast that Mob1 and Mob2 are required for the activity of the NDR-related kinases Dbf2 and Cbk1, respectively (26, 27). To test whether hMOB1 also plays a role in NDR activation, we cotransfected myc-hMOB1 or the empty vector with HA-NDR1 into COS1 cells. The cells were treated with the PP2A-inhibitor OA. OA is, to date, the only known potent activator of NDR *in vivo* (20, 21) and it has been shown that yeast Mob1 becomes phosphorylated after OA treatment (27). The kinase activity of immunoprecipitated HA-NDR1 was stimulated 35-fold by a 45-min 1 μM OA treatment (Fig. 1A). Coexpression of myc-hMOB1 induced a further two- to three-fold increase in NDR kinase activity (Fig.1A). At the 45 min OA time point, HA-NDR1 coexpressed with myc-hMOB1 was stimulated 100-fold compared to the control. This indicates a role for hMOB1 in NDR activation. To test whether hMOB1 acts directly on NDR, we performed *in vitro* kinase assays of bacterially expressed SHP-NDR2 in the presence of GST-hMOB1 or GST. We used NDR2 for in vitro experiments because it can be readily produced in sufficient amounts. NDR2, which is 86% identical to NDR1, has been shown to be regulated in the same way as NDR1 (34). Furthermore, we confirmed with NDR1 produced in Sf9 cells that wild-type NDR1 behaves *in vitro* similar to NDR2 with respect to hMOB1 (data not shown). GST-hMOB1 stimulated SHP-NDR2 autophosphorylation about two-fold (data not shown). Furthermore, SHP-NDR2, which was pre-autophosphorylated in the presence of GST-hMOB1, has an up to sixfold higher kinase activity against the NDR substrate peptide than SHP-NDR pre-autophosphorylated in the presence of

GST (Fig. 1B). These results show that hMOB1 has a direct, positive effect on NDR kinase activity. We tested the effect of hMOB1 on the phosphorylation state of the NDR phosphorylation sites Thr-74, Ser-281 and Thr-444 (Thr-75, Ser-282 and Thr-442 in the case of NDR2; the phosphorylation sites of NDR2 were recognised by phosphospecific antibodies generated against the corresponding phosphorylation sites of NDR1). SHP-NDR2 phosphorylation on the autophosphorylation sites Ser-282 and Thr-75 was slightly increased, whereas Thr-442 of SHP-NDR2, which is known to be targeted by an upstream kinase *in vivo*, showed no autophosphorylation (Fig. 1B).

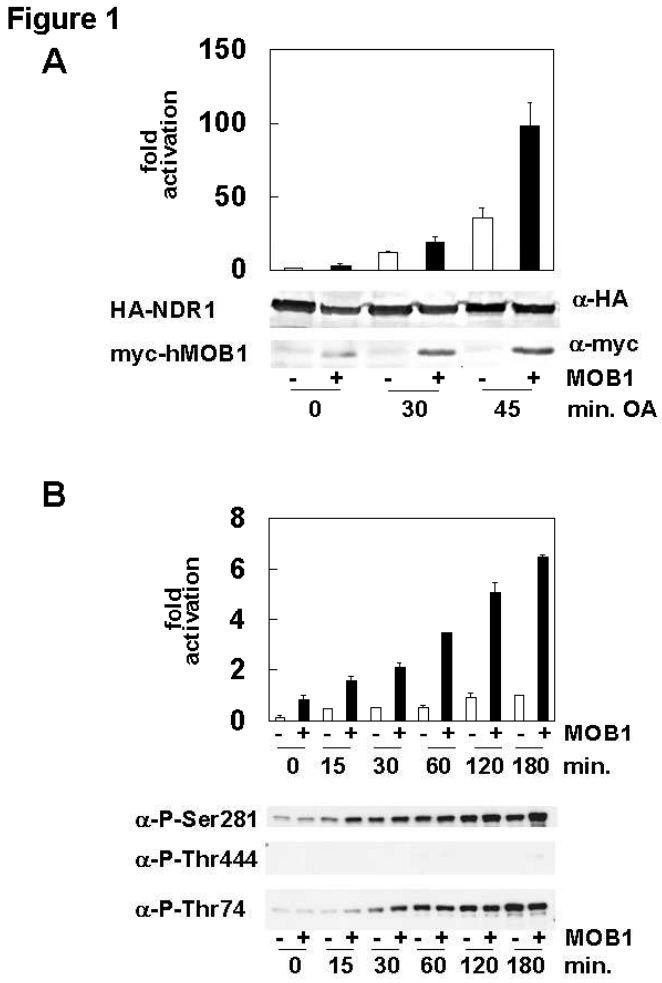

Fig. 1. hMOB1 stimulates NDR kinase activity. A) *HA-NDR was cotransfected with myc-hMOB1 or the empty vector and treated for the indicated times with 1 μM OA. 12CA5 immunoprecipitates were then assayed for kinase activity. Also shown is the expression of HA-NDR and myc-hMOB1 in the total cell lysates.* ***B)*** *GST-hMOB1 or GST were added to bacterially-expressed SHP-NDR2 and autophosphorylation reactions performed for the indicated times. After autophosphorylation, half of the reaction was added to a mixture containing* ^{32}*P-ATP and NDR substrate peptide to perform kinase assays. The other half was stopped with SDS sample buffer and resolved on a 10% SDS gel for western blots with phosphospecific antibodies.*

hNDR1 interacts with hMOB1 – To investigate whether hMOB1, the closest relative of yeast MOB2 and MOB1 in mammals, is able to interact with NDR1, epitope-tagged NDR1 and hMOB1 were cotransfected into COS1 cells. In co-immunoprecipitation of myc-hMOB1 with HA-NDR1 (Fig. 2A), as well as in co-immunoprecipitation of HA-NDR with myc-hMOB1 (Fig. 2B), hMOB1 associated with NDR1. The protein level of myc-hMOB1 was dramatically increased upon OA stimulation, whereas myc-hMOB1 transfected without NDR1 was present at similar levels in OA-treated and untreated cells. We did not address the molecular basis of this phenomenon. The kinase dead mutant with mutated catalytic lysine in the ATP binding site, K118A, was still able to interact with hMOB1 after OA stimulation (Fig. 2A). Thus, the interaction was not dependent on NDR kinase activity. Furthermore, we tested whether the two important in vivo phosphorylation sites of NDR, Ser-281 and Thr-444, as well as a recently identified in vitro autophosphorylation site, Thr-74, play a role in NDR–MOB interaction. The NDR mutants S281A and T444A still interacted with hMOB1 after OA stimulation, while the T74A mutant showed almost complete absence of interaction with hMOB1 (data not shown).

NDR-hMOB1 interaction depends on OA-induced modification on hMOB1, while phosphorylation of NDR is not required – Myc-hMOB1 interacted with HA-NDR1 in OA-treated COS1 cells, but it was not clear whether OA acted just by increasing the myc-hMOB1 protein level or whether OA-induced modification of NDR1, hMOB1 or both promoted the interaction. To address this question, we transfected HA-NDR1 and myc-hMOB1 separately into COS1 cells and stimulated them with 1 μM OA or left them unstimulated. We then pooled the

lysates (NDR1 ± OA with hMOB1 ± OA) and immunoprecipitated with α-HA antibody. In the two combinations containing myc-hMOB1 of OA-treated cells, myc-hMOB1 strongly associated with HA-NDR, regardless of whether the HA-NDR was expressed in OA-treated or unstimulated cells. Myc-hMOB1 from unstimulated cells showed only a weak interaction with HA-NDR from both unstimulated and OA-stimulated cells (Fig. 2C). Thus, OA-induced phosphorylation of NDR is not required for interaction but hMOB1 modification is necessary. Furthermore, GST-hMOB1 was phosphorylated by immunoprecipitated HA-NDR. The phosphorylation of GST-hMOB1 increased three- to fourfold when HA-NDR was immunoprecipitated from OA-stimulated COS1 cells. However, phosphorylation of GST-hMOB1 was also observed when kinase dead HA-NDR was immunoprecipitated (Fig. 2D). We conclude that a kinase that co-immunoprecipitates with NDR is able to phosphorylate hMOB1.

Figure 2

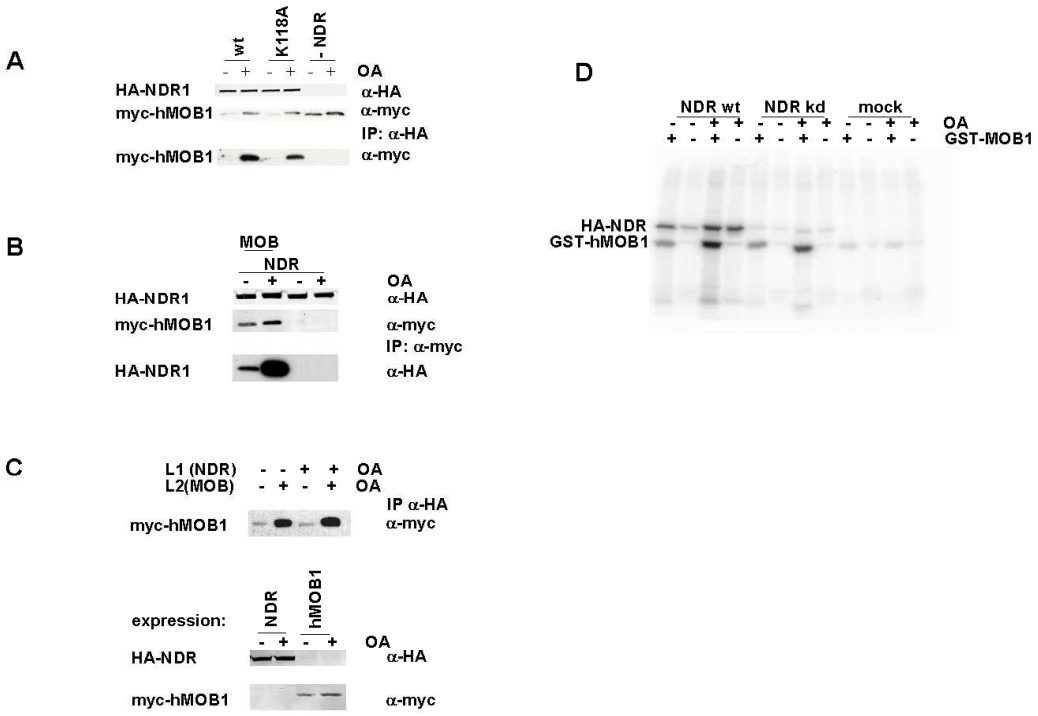

Fig. 2. NDR interacts with hMOB1. A, B) *HA-NDR, wild type and kinase dead (kd), and myc-hMOB1 were cotransfected into COS1 cells and the cells treated with OA prior to lysis.* ***A)*** *HA-NDR was immunoprecipitated and the co-immunoprecipitated myc-hMOB1 detected.* ***B)*** *myc-hMOB1 was immunoprecipitated and the co-immunoprecipitated HA-NDR detected.* ***C)*** *HA-NDR and myc-hMOB1 were transfected separately into COS1 cells and the cells treated with OA or solvent alone prior to lysis. Lysates of OA-stimulated and unstimulated cells containing HA-NDR1 and lysates of OA-stimulated and unstimulated cells containing myc-hMOB1 were combined. HA-NDR was immunoprecipitated from these combined lysates and co-immunoprecipitated myc-hMOB1 was detected.* ***D)*** *HA-NDR wild type and the kinase dead mutant K118A were transfected into COS1 cells and the cells treated for 60 min with 1 μM OA or the solvent alone prior to lysis. HA-NDR was immunoprecipitated out of 1 mg lysate protein. HA-NDR kinase reactions were performed for 2 h with 10 μM GST-hMOB1 or GST instead of the NDR substrate peptide. The reactions were resolved on a 10% SDS-PAGE and the incorporated ^{32}P visualised with a PhosphorImager.*

The highly conserved N-terminal domain of NDR is required for kinase activation – The N-terminal regulatory domain of NDR kinase is highly conserved in the closest relatives of NDR throughout the eukaryotic world (Fig. 3A). Several residues are completely invariant throughout evolution from single cell organisms to humans. This prompted us to test the functional significance of these residues with respect to NDR kinase activity. Mutations of highly conserved residues induced strong inhibition of OA-stimulated kinase activity (Fig 3B). The first part of the N-terminal domain covering amino acids 1 to 33 and containing a predicted β-sheet in hNDR proved to be important for kinase activation. Deletion of the first 30 amino acids completely abolished kinase activation (data not shown). Point mutations in this region strongly reduced kinase activity: mutation of Thr-16, Glu-18 and Glu-28 reduced activity to about 40%, whereas mutation of Lys-24 and Tyr-31 reduced activity to about 20%. Mutation to alanine of Arg-41, Arg-44 or Leu-48, all of which lie in a predicted first α-helix covering the amino acids 40-55 and are situated close together on the same side of the predicted α-helix, reduced kinase activity to below 20% of wild-type activity. Mutation of residues in a predicted second α-helix situated in the previously described S100B binding region of NDR and covering amino acids 60-80 also led to inhibition of kinase activity. Mutation of Lys-72, Glu-73, Thr-74, Arg-78 and Leu-79 to alanine reduced kinase activity to 20% or lower. Taken together, the results imply that the high conservation of the N-terminal domain, further termed SMA (S100B and MOB Association) domain, is due to an absolute requirement of the conserved residues for proper kinase function, either by ensuring the correct structural conformation of the protein or being directly involved in the binding to interacting proteins.

Figure 3

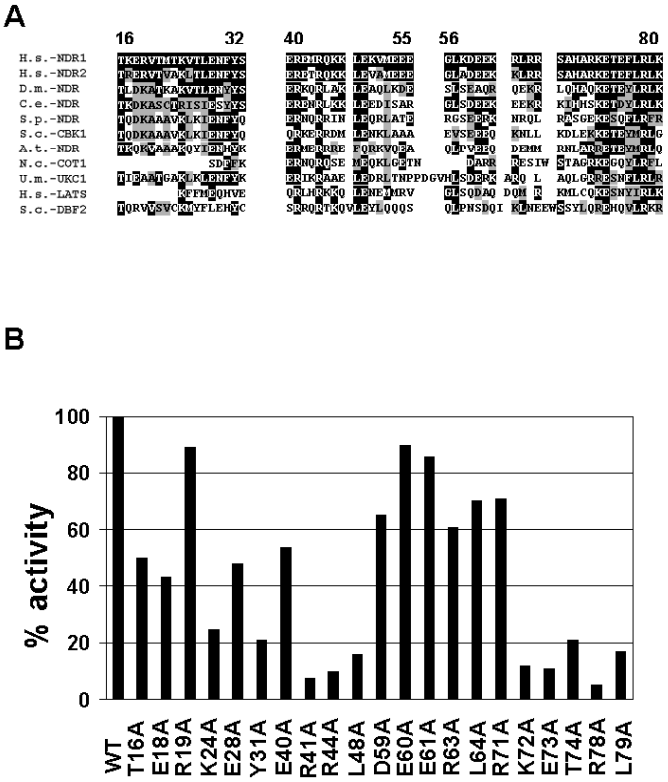

Fig. 3. The N-terminal SMA domain of NDR is essential for kinase activity. A) *Alignment of the N-terminal domain of NDR and its relatives. Numbering based on NDR1.* ***B)*** *Relative kinase activities of HA-NDR1 point mutants from OA-stimulated COS1 cells. The kinase activities of HA-NDR and the indicated mutants were measured. The data are a summary of three experiments (mutants amino acids 16-31, 40-60 and 61-79). NDR wild type was stimulated 20- to 30-fold by OA in these experiments. The activities of the NDR mutants were compared to the activity of wild-type NDR in each experiment. Similar expression levels of NDR wild type and mutants were confirmed in each experiment by HA-western blotting.*

The SMA domain is required for NDR-MOB interaction – Thr-74, which was previously shown to be important for NDR kinase activity and interaction with S100B, also turned out to be required for NDR–hMOB1 interaction in COS1 cells. Therefore, we investigated the involvement of the conserved residues of the SMA domain in NDR–hMOB1 interaction. Cotransfection of HA-tagged NDR mutants and myc-hMOB1 and subsequent co-immunoprecipitation of myc-MOB with HA-NDR revealed that several of the conserved residues important for kinase activation are also required for NDR–hMOB1 interaction (Fig. 4A-C). Tyr-31, Arg-41, Thr-74 and Arg-78 seem to be absolutely required for interaction, while the Lys-24, Arg-44 and Leu-79 mutants displayed reduced interaction. Only three of the mutants with a strongly reduced kinase activity (Arg-48, Lys-72, Glu-73) showed no decrease in ability to bind hMOB1.

Figure 4

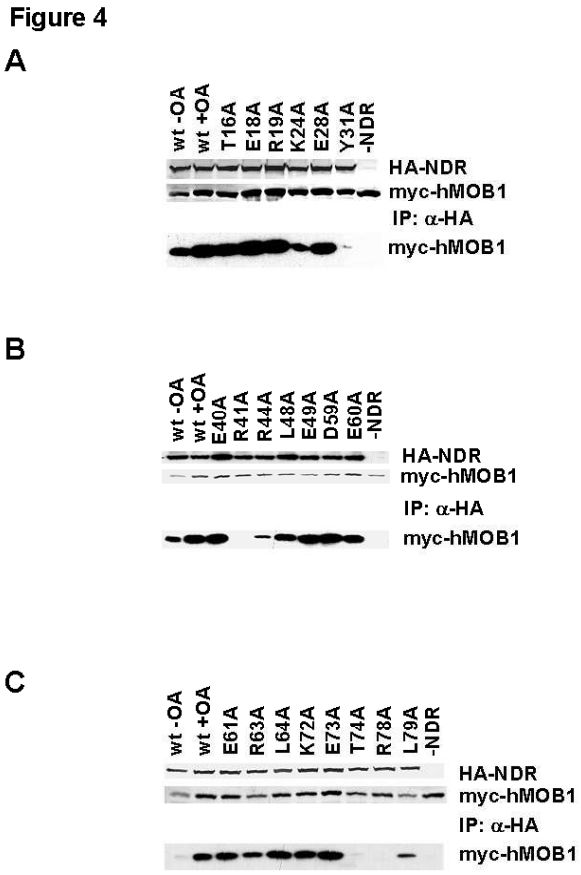

Fig. 4. The SMA domain of NDR is required for NDR–hMOB1 interaction. *HA-NDR N-terminal point mutants and myc-hMOB1 were cotransfected into COS1 cells that were treated with OA prior to lysis. HA-NDR mutants were immunoprecipitated and co-immunoprecipitated myc-hMOB1 was detected. A) Point mutants amino acids 16-31, B) point mutants amino acids 40-60, C) point mutants amino acids 61-79.*

Tyr-32 of NDR2 kinase is essential for activation by hMOB1 in vitro – The residues Tyr-31, Arg-41, Arg-44, Thr-74 and Arg-78 of NDR1 were shown to be important for kinase activation and interaction with hMOB1 in COS1 cells. We examined whether these residues are involved directly in the interaction of NDR and hMOB1 or indirectly by influencing interactions with other proteins in the cells. We used bacterially-produced SHP-NDR2, wild type and the mutants Y32A, RR4245AA, E74A, T75A and R79A (corresponding to the NDR1 mutants Y31A, RR4144AA, E73A, T74A and R78A), together with GST-hMOB1 protein to perform in vitro kinase assays to measure the direct effect of hMOB1 on NDR kinase activity (Fig. 5A). Mutation of Glu-74, a residue that affects kinase activity but not interaction with hMOB1 in COS1 cells, led to reduced kinase basal activity *in vitro*. This very highly conserved Glu-74 seems to have an important intrinsic role for the function of NDR kinase, although it is not involved in the binding to hMOB1. Mutation of residues Arg-42 and Arg-45, which lie on the same side of a predicted α-helix and whose mutation affects NDR–hMOB1 interaction as well as kinase activation in COS1 cells, led to a reduction in kinase activity *in vitro* but did not completely abolish the activation by hMOB1. NDR with mutated Tyr-32 showed an intact basal kinase activity but was not activated by hMOB1 at all *in vitro*, pointing to an important role for this residue in the direct interaction with hMOB1. Activation of the T75A and the R79A mutants by hMOB1 was not different to wild-type NDR, suggesting that Thr-75 and Arg-79 are not directly involved in binding to hMOB1 and do not affect the kinase activity of NDR *per se*. Furthermore, pull-down assays showed that NDR2 with mutated Tyr-32 does not bind to hMOB1, while the binding of NDR2 with mutated Arg-42 and Arg-45 is not abolished (Fig. 5B).

Figure 5

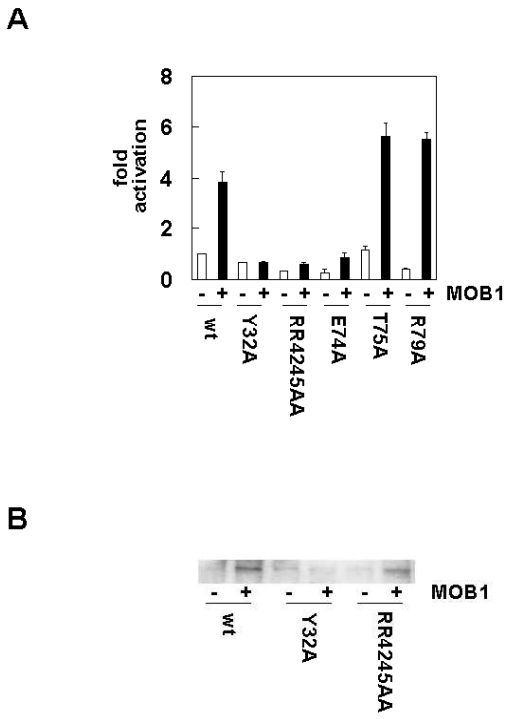

Fig. 5. Influence of SMA domain mutants on the in vitro kinase activation of NDR by hMOB1 and the NDR–hMOB1 interaction. A) *1 µg wild-type SHP-hNDR2 and the indicated mutants was pre-autophosporylated in the presence of 10 µM GST-hMOB1 or GST for 2 h. The reactions were subsequently mixed with γ^{32}P-ATP and NDR substrate peptide and incubated for 60 min to determine the NDR peptide kinase activity.* ***B)*** *25 µg GST or GST-hMOB1 was incubated with 20 µl gluthathione Sepharose for 2 h at 4°C. The beads were washed three times with TBS and 5 µg SHP-NDR wild type and mutants were then added and the mixture incubated for 3 h at 4°C. The beads were washed five times with TBS, resuspended in 30 µl 1x SDS sample buffer and the samples resolved by 12% SDS PAGE. Bound NDR was detected by western blotting using the anti-NDR antibody.*

The insert in the catalytic domain acts as an autoinhibitory sequence – A common feature of the NDR family of kinases is an insert in the catalytic domain of 30-60 amino acids between subdomains VII and VIII. The insert sequence is not highly conserved, but contains in all cases many positively charged amino acids. Therefore, we tested the importance of these positive residues for NDR activity and NDR–hMOB1 interaction. Mutating the sequence amino acids 265-KRKAETWKRNRR-276 to amino acids 265-AAAAETWAANRR-276 increased both kinase activity and phosphorylation of Thr-74, Ser-281 and Thr-444 in COS1 cells (Fig. 6A, B), but did not affect NDR–MOB binding (data not shown). To test whether this insert sequence has an autoinhibitory function, we produced recombinant SHP-NDR2 insert mutant and tested its *in vitro* activity. NDR with the mutated insert sequence had a four- to six-fold elevated basal kinase activity, similar to hMOB1 stimulated NDR2, proving that the insert sequence is autoinhibitory. Moreover, NDR2 with a mutated autoinhibitory sequence (AIS) was only slightly, about 1.5-fold, stimulated by hMOB1. Furthermore, we examined the effect of the combined mutation of Tyr-32 and the AIS. Mutation of Tyr-32, which abolishes binding of hMOB1 to NDR, did not affect the activity of the AIS-mutant NDR2, and the AIS Y32A mutant was not activated by hMOB1 (Fig. 6C). Altogether, these results indicate that hMOB1 binding induces the release of the autoinhibition caused by the AIS.

Figure 6

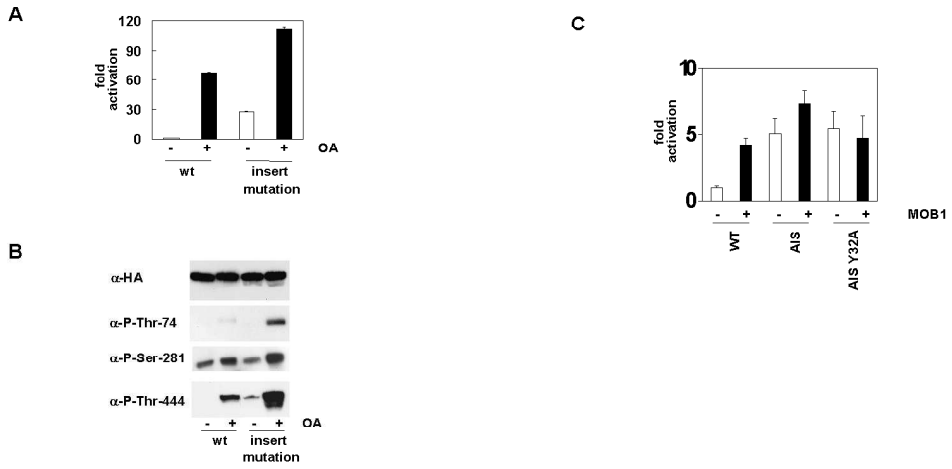

Fig. 6. Mutation of the insert in the catalytic domain activates NDR kinase. A) *Kinase activity of HA-NDR insert mutant. The amino acid sequence 265 KRKAETWKRNRR 276 was mutated to 265 AAAAETWAANRR 276. HA-NDR, wild type and insert mutant were expressed in COS1 cells and the cells treated with OA or solvent alone, as indicated. HA-NDR was immunoprecipitated and NDR kinase activity against the NDR substrate peptide measured.* ***B)*** *Western blots showing the expression levels (anti-HA western blot) and the phosphorylation states of Thr-74, Ser-281 and Thr-444.* ***C)*** *In vitro kinase activity of NDR insert mutant. 1 μg SHP-NDR2 wild type, insert mutant (AIS=autoinhibitory sequence) and insert mutant with mutated Tyr-32 were pre-autophosphorylated in the presence of 10 μM GST-hMOB1 or GST for 2 h. Reactions were mixed with γ-^{32}P-ATP and NDR substrate peptide and incubated for 60 min to determine NDR kinase activity.*

The Ca^{2+} chelator BAPTA-AM reduces NDR-MOB interaction in cells, but the action of hMOB1 on NDR in vitro is Ca^{2+}-independent – Previously, we showed that treatment of COS1 cells with the Ca^{2+} chelator BAPTA-AM reduces OA-induced NDR kinase activation (21). Therefore, we tested whether treatment of COS1 cells with BAPTA-AM influences NDR–hMOB1 interaction. In co-immunoprecipitation of myc-hMOB1 with HA-NDR of BAPTA- and OA-treated cells, BAPTA-AM reduced NDR–hMOB1 interaction corresponding to the observed reduction in NDR kinase activation (Fig. 7A). Therefore, we tested whether Ca^{2+} influences the action of hMOB1 on NDR *in vitro.* Addition of Ca^{2+} to NDR *in vitro* kinase assays, in the presence or absence of GST-hMOB1, had no effect on NDR kinase activity (Fig. 7B). Thus Ca^{2+} has no direct influence on NDR–hMOB1 interaction.

Figure 7

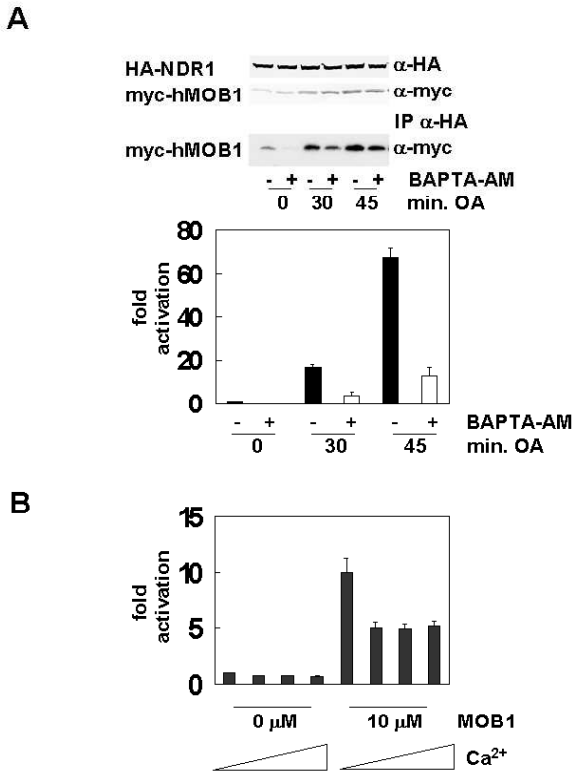

Fig.7. Ca^{2+} depletion of COS1 cells reduces NDR–hMOB1 interaction but Ca^{2+} has no direct role in NDR–hMOB1 interaction A) *HA-NDR1 and myc-hMOB1 were cotransfected into COS1 cells and the cells treated with BAPTA-AM and OA or the solvents alone, as indicated. HA-NDR was immunoprecipitated and the co-immunoprecipitated myc-hMOB1 detected. The kinase activity of the HA-NDR immunoprecipitates was measured in a peptide kinase assay.*

B) *1 μg SHP-NDR2 was pre-autophosphorylated for 2 h in the presence of 10 μM GST-hMOB1 or GST at 0, 10, 100 and 1000 μM Ca^{2+}. Zero Ca^{2+} was achieved by the addition of 2 mM EGTA to the kinase reactions. The reactions were subsequently mixed with γ^{32}P-ATP and NDR substrate peptide and incubated for a further 60 min to determine the peptide kinase activity of NDR.*

Discussion

We found that NDR kinase is activated by and interacts with hMOB1 *in vitr*o and in COS1 cells. MOB proteins, similar to NDR family kinases, are highly conserved throughout the eukaryotic world. hMOB1 is the closest relative of yeast Mob1 and Mob2. The yeast NDR family kinases Dbf2 and Cbk1 were both shown to interact with yeast Mob1 and Mob2. The finding that hMOB1 activates and interacts with NDR supports the existence of a highly conserved signalling pathway. The interaction of NDR and hMOB1 in COS1 cells increased when cells were treated with OA. We have shown that interaction of NDR1 and hMOB1 in cell lysates depends on an OA-induced modification of hMOB1 but not of NDR. A possible explanation for this is that hMOB1 is sequestered by interaction with another protein and hMOB1 is released upon OA treatment and interacts with NDR. Therefore, the observed activation of NDR by OA may not be due solely to direct inhibition of Ser-281 and Thr-444 dephosphorylation and activation of the pathway leading to Thr-444 phosphorylation, but also to an increase in hMOB1 interaction with NDR. The observed phosphorylation of GST-hMOB1 by immunoprecipitated wild-type and kinase dead HA-NDR1 suggests that OA stimulates a kinase that phosphorylates hMOB1 and is in a complex with NDR. It is conceivable that this phosphorylation is responsible for the binding of NDR to hMOB1 in COS1 cells. There is now a need to identify the phosphorylation sites on hMOB1 and the kinase that phosphorylates hMOB1. We showed previously that NDR activation depends on Ca^{2+} (21). Treatment of COS1 cells with the Ca^{2+} chelator BAPTA-AM led to a decrease in NDR–hMOB interaction. However, Ca^{2+} had no effect on hMOB1-stimulated NDR activity *in vitro*. Therefore, Ca^{2+}

may be required for MOB modification *in vivo* but it plays no role in the direct activation of NDR by MOB.

Several residues within the N-terminal SMA domain (S100B and MOB association domain) of NDR are important for NDR−hMOB1 interaction and for the kinase activation *in vivo*. On the other hand, only one of these residues, Tyr-32, proved to be important for interaction with and activation by hMOB1 *in vitro*. In contrast, it has been shown by NMR studies that *X. laevis* MOB1 interacts with a synthetic peptide covering the S100B binding region of NDR (35). This region also contains residues Thr-74 and Arg-78, which are important for NDR activation and interaction with hMOB1 *in vivo*. Furthermore, the previously resolved crystal (31) and NMR (35) structures of hMOB1α and *X. laevis* MOB1, respectively, revealed that MOB1 has a negatively charged, exposed potential interaction surface. Thus, it is likely that positively charged residues of the NDR SMA domain, such as Arg-78, are involved in the interaction with hMOB1 but that its mutation is not sufficient to disrupt interaction with NDR under *in vitro* conditions. The residues Arg-41 and Arg-44, which lie on the same side of a predicted α-helix, may also participate in the interaction but their mutation is not sufficient to disrupt the interaction *in vitro*. Mutation of Tyr-32, however, might disrupt the overall structure of the SMA domain, thereby disabling the interaction with hMOB1, or Tyr-32 of NDR might interact directly with hMOB1.

Although the sequence of the insert in the kinase catalytic domain between subdomains VII and VIII is not well conserved between NDR and the yeast kinases Cbk1 and Dbf2, they all have a sequence with a high basic amino acid content. Since this sequence is located just in front of the activation segment phosphorylation site, the question arises of whether it has a regulatory role. Mutation of the basic residues in this insert led to kinase activation both in cells

and *in vitro*, showing that the sequence acts autoinhibitory. When NDR with mutated AIS was mutated on Tyr-32, a residue essential for NDR–hMOB1 interaction, unstimulated kinase activity was not affected. This points to a new mechanism of kinase activation: binding of MOB may induce a conformational change that leads to release of the autoinhibition caused by the AIS. Crystallographic studies of MOB-bound and –unbound NDR would be required to test this model. It is noteworthy that phosphorylation of the hydrophobic motif site Thr-444 of the NDR AIS mutant in COS1 cells also increased. Thus, the release of autoinhibition also facilitated phosphorylation by the hydrophobic motif upstream kinase. In accordance with this, it was suggested previously that binding of yeast Mob1 to Dbf2 enables the Ste20-like kinase Cdc15 to phosphorylate Dbf2 (27).

It has been shown that the autoinhibitory sequence acts as a nuclear localisation signal in COS1 cells in the case of NDR1 (17). In yeast, Mob2 is important for the localisation of the NDR relative Cbk1 (28, 29). It is possible that the conformational change induced by hMOB1 also influences NDR localisation and this will be addressed in future studies. It will also be interesting to examine whether other members of the MOB family (hMOB1 α,β, hMOB2, hMOB3 α,β,γ) act as kinase activators *in vivo*. During preparation of this article, it has been reported that hMOB2 interacts with NDR1 and NDR2 from Jurkat cells and that hMOB2 stimulates NDR kinase activity (36).

S100B, a previously described activator of NDR, is constitutively bound to NDR in cells independent of OA stimulation (21). S100B may constitutively maintain the correct conformation of the SMA domain. It has been shown recently that the NDR-derived S100B-binding peptide adopts its helical conformation after binding to S100B (37). A high concentration of S100B in certain cell types, for example

melanoma cells (18), may thus lead to constitutively elevated NDR activity. MOB proteins, in contrast, may transmit a signal by fluctuation of the MOB protein level during the cell cycle, as was reported for S. cerevisiae Mob1 (38), and/or by post-translational modification of MOB that promotes the interaction with NDR, as we have suggested for the mechanism of OA-induced NDR kinase activation.

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Footnotes:

[1] The abbreviations used are: NDR, Nuclear Dbf2 related and for abbreviations of other kinase names see (1); BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl)ester; GST, gluthathione S-transferase; HA, hemagglutinin; DDT, dithiothreitol; OA, okadaic acid; SMA, S100B and MOB association; AIS, autoinhibitory sequence, wt, wild-type; PP2A, protein phosphatase 2A; PCR, polymerase chain reaction; ECL, enhanced chemo luminescence

[2] Forrer, P., Chang, C., Ott, D., Wlodawer, A. and Plückthun, A. *J. Mol. Biol.*; submitted.

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7.2. Mechanism of Ca^{2+} -mediated regulation of NDR1 and NDR2 through autophosphorylation and phosphorylation by an upstream kinase

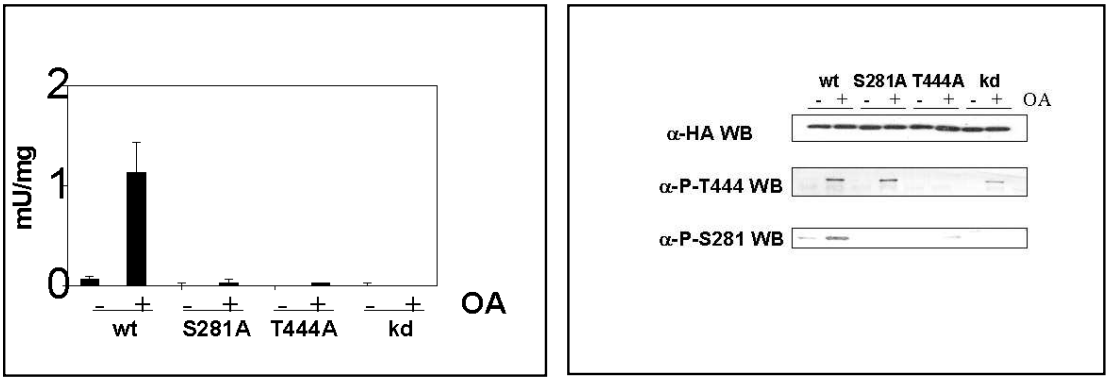
In this section, I present the results which were on the basis of the publications by Tamaskovic et al and Stegert et al (see section 10.).

7.2.1. NDR1 is phosphorylated on the activation segment site Ser-281 by autophosphorylation, while the hydrophobic motif site Thr-444 is phosphorylated by an upstream kinase

NDR1 kinase is efficiently phosphorylated in vivo upon okadaic acid treatment on the activation segment site Ser-281 and the hydrophobic motif site Thr-444.

To examine the mode of phosphorylation of these phosphorylation sites, HA-NDR phosphorylation site mutants, kinase dead HA-NDR K118A and wild type HA-NDR were transfected into COS-1 cells. The COS-1 cells were treated with 1μM OA prior to lysis. HA-NDR, wild type and the mutants were immunoprecipitated and the kinase activity measured. Mutation of Ser-281 as well as of Thr-444 reduces NDR kinase basal activity as well as the OA-stimulated activity (A). Western blot analysis with phosphospecific antibodies revealed that Thr-444 is phosphorylated independent of NDR kinase activity; the kinase dead NDR mutant is phosphorylated on Thr-444. On the other hand, Ser-281 phosphorylation is dependent on NDR kinase activity; the kinase dead mutant is not phosphorylated on Ser-281 (B). We can conclude that the activation segment site Ser-281 is phosphorylated by autophosphorylation, while the hydrophobic motif site Thr-444 is phosphorylated by an upstream kinase.

A) B)

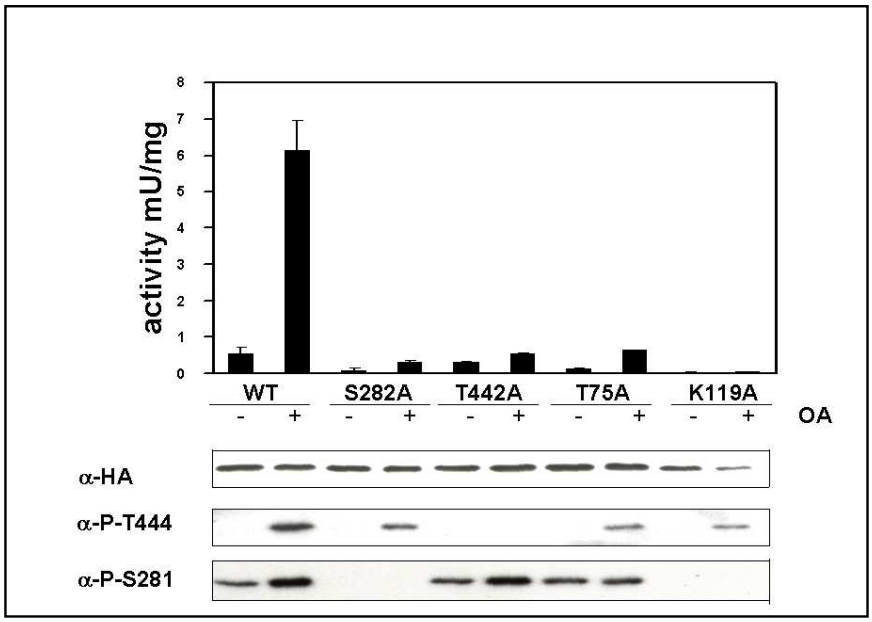


NDR1 is phosphorylated on the activation segment site Ser-281 by autophosphorylation, while the hydrophobic motif site Thr-444 is phosphorylated by an upstream kinase in vivo. *A) COS-1 cells expressing either wild-type HA-NDR1 or the indicated mutants were treated for 1 h with 1 μM OA or with solvent alone. HA-tagged NDR kinase mutants were then immunoprecipitated (of 250 μg detergent extracts) and assayed for kinase activity by a peptide kinase assay. B) 1μg of protein extracts from transfected COS-1 cells was immunblotted with 12CA5 to verify similar expression level of each HA-NDR construct (top panel). For analysis of the phosphorylation status, 12CA- immunoprecipitated HA-NDR1 variants were analysed by immunoblotting with phosphospecific antibodies directed against phosphorylated Ser-281 or phosphorylated Thr-444 (second and third panel).*

7.2.2. NDR2 is phosphorylated on the activation segment site Ser-282 by autophosphorylation, while the hydrophobic motif site Thr-444 is phosphorylated by an upstream kinase

The kinase domain of hNDR2 is 91% identical to hNDR1. Here we tested if the mode of activation of NDR2 is similar to that of NDR1. HA-NDR2, wt , phosphorylation site mutants and kinase dead K119A mutant, were transfected into COS-1 cells. The COS-1 cells were treated with 1μM OA prior to lysis. HA-NDR, wild type and the mutants were immunoprecipitated and the kinase activity

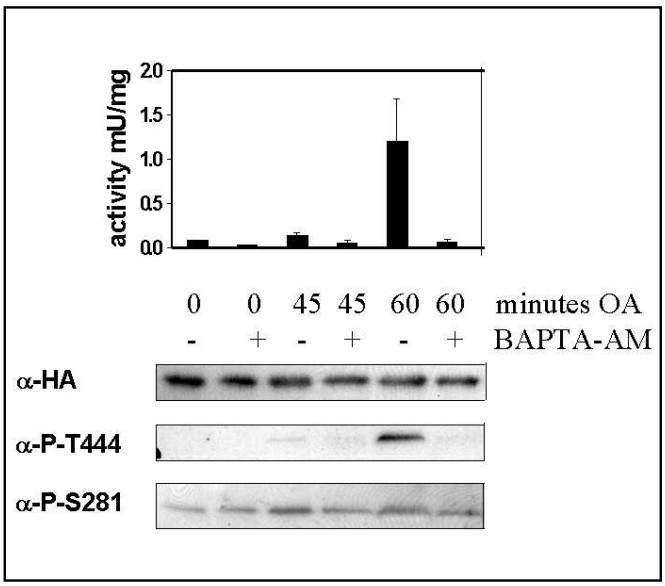
measured and the phosphorylation state of Ser-282 and Thr-442 determined. The results lead to the same conclusions as for NDR1: The activation segment phosphorylation site is an autophosphorylation site, while the hydrophobic motif site is phophorylated by an upstream kinase.



NDR2 is phosphorylated on the activation segment site Ser-282 by autophosphorylation, while the hydrophobic motif site Thr-442 is phosphorylated by an upstream kinase in vivo. *COS-1 cells expressing either wild-type HA-NDR2 or the indicated mutants were treated for 1 h with 1 μM OA or with solvent alone. HA-tagged NDR kinase mutants were then immunoprecipitated (of 250 μg detergent extracts) and assayed for kinase activity by a peptide kinase assay(top) 1μg of protein extracts from transfected COS-1 cells was immunblotted with 12CA5 to verify similar expression level of each HA-NDR construct. For analysis of the phosphorylation status, 12CA-immunoprecipitated HA-NDR1 variants were analysed by immunoblotting with phosphospecific antibodies directed against phosphorylated Ser-281 or phosphorylated Thr-444 (these antobodies recognise the corresponding phosphorylated residues Ser-282 and Thr-442 of NDR2).*

7.2.3. The Ca^{2+} -chelator BAPTA-AM reduces NDR activity andphosphorylation on Ser-281 and Thr-444

Previously, it has been shown that NDR kinase is activated by Ca^{2+} -dependent binding of S100B protein in vitro (Millward et al, 1998). Here we examined the importance of intracellular Ca^{2+} on NDR activity and Ser-281 and Thr-444 phosphorylation. For this purpose, transfected COS-1 cells were treated with the membrane permeable agent BAPTA-AM, which is freely taken up into cells were it is hydrolysed by esterases and trapped intracellularly as the active and membrane impermeable Ca^{2+} -chelator BAPTA. The result show that treatment with 50 μM BAPTA-AM dramatically reduces OA induced NDR activation. The phosphorylation of the activation segment site Ser-281, but also of the hydrophobic motif site Thr-444 is reduced. Therefore, intracellular Ca^{2+} is important for the autophosphorylation of Ser-281 as well as for the phosphorylation of Thr-444 by an upstream kinase.



Phosphorylation ofr both Ser-281 and Thr-444 is dependent on intracellular Ca^{2+}.

HA-NDR1- expressing COS-1 cells were treated for 1h as indicated with 1 μM OA, 50 μM BAPTA-AM or the solvents alone. 12CA5 immunoprecipitates (of 200 μg of protein extracts) were then assayed by peptide kinase assay for NDR kinase activity (top). Protein extracts were immunblotted with the 12CA5 antibody to verify equal expression levels. For the analysis of the phosphorylation status, 12CA5-immunoprecipitated HA-NDR (of 200 μg of protein extract) was analysed by immunoblotting with the the phosphospecific antibodies directed against P-281 or P-T444.

8. Discussion:

8.1. Regulation of NDR kinases

It has been shown that human NDR kinases possess two phosphorylation sites that are important for kinase activation (Millward et al, 1999). These two sites are the activation segment site that is phosphorylated by autophosphorylation (Tamaskovic et al, 2003) and the hydrophobic motif site that is phosphorylated by a Ste20 like kinase (Stegert et al, manuscript in preparation). Human NDR kinases are dephosphorylated by the action of protein phosphatase 2A (PP2A) (Millward et al, 1999). This phosphorylation sites are highly conserved among all members of the NDR kinase family. Therefore it is likely that all other members of the NDR family are regulated by phosphorylation/dephosphorylation on these two sites. Indeed, it has been shown for the budding yeast relative Dbf2 that these two sites are phosphorylated, essential for activity, and that the phosphorylation depends on the Ste20 like kinase Cdc15 (Mah et al, 2001).

Treatment of cells with the PP2A inhibitor okadaic acid efficiently stimulates NDR kinase activity, but to date, nothing is known about physiological stimuli that induce NDR kinase activation. The budding yeast NDR kinases Dbf2 and Dbf20 are activated in a cell cycle dependent manner, the activity peaks after the metaphase to anaphase transition (Toyn et al, 2004). Interestingly, human NDR kinase activity is cell cycle dependent as well: Activity and hydrophobic motif phosphorylation increases at the G1/S boundary and persists until mitosis and cytokinesis. (Tamaskovic et al, manuscript in preparation). Therefore, it is likely that NDR kinases are not activated by an external stimulus, but rather by internal signals that are generated during the cell cycle.

NDR kinase activation depends on internal Ca^{2+}, and Ca^{2+}-binding protein S100B activates NDR kinase in vitro (Millward et al, 1998, Tamaskovic et al, 2003). The

regulatory role of Ca^{2+} in vivo is not absolutely clear. Overexpression of S100B in COS-1 cells does not activate NDR. Treatment of COS-1 cells with a Ca^{2+}-ionophore stimulates NDR activity only slightly while Ca^{2+}-depletion of COS-1 cells leads to a dramatic reduction of NDR kinase activity and Ser-281 and Thr-444 phosphorylation. S100B is in vivo bound to not activated as well as activated NDR. Nevertheless, endogenous NDR from certain melanoma cell lines with elevated S100B-levels shows a higher kinase activity (Millward et al, 1998). Therefore, S100B may be necessary to keep NDR in a correct conformation, so the higher activity of NDR from S100B positive melanomas may reflect a higher level of properly folded NDR, while S100B/Ca^{2+} may not fulfil a short term regulatory role. To date, there is nothing known about a role for Ca^{2+} and S100B related proteins for the regulation of NDR family kinases in other organisms.

Mob proteins bind to and activate the NDR family kinases Cbk1 and Dbf2 in budding yeast and Orb6 and Sid2 in fission yeast. Mob proteins are, like NDR family kinases, highly conserved throughout the eukaryotic world. Human NDR kinase is activated by hMOB1, and hMOB1 binds to the N-terminal domain of NDR. The interaction with MOB apparently induces a conformational change, which induces the release of an autoinhibition. The autoinhibition is caused by an autoinhibitory sequence situated in the insertion in the catalytic domain. This insertion, which is high in basic amino acids, is characteristic for all NDR family kinases. The activation mechanism of NDR kinases by Mob proteins appears to be highly conserved during evolution.

The budding yeast kinase Cbk1 interacts with the Ste20 like kinase Kic1 (Jorgensen et al, 2002), and Dbf2 is phosphorylated by the Ste20 like kinase Cdc15. The phosphorylation of Dbf2 by Cdc15 depends on budding yeast Mob1.

(Mah et al, 2001). It has been shown in our lab that the Ste20-like kinase MST3 phosphorylates NDR2 kinase at the hydrophobic motif site Thr-442 (Stegert et al, manuscript in preparation).

8.2. Function of NDR kinase:

Members of the NDR kinase family in unicellular organisms are involved in the regulation of cell morphology and the cell cycle (see introduction). The function of the NDR kinase orthologues in multicellular organisms has been previously investigated in D. melanogaster and C. elegans.

8.2.1. D. melanogaster

The D. melanogaster orthologue, TRC, encoded by the tricornered (trc) gene, is 70% identical and 79% similar to human NDR kinase. Trc mutations are organismal recessive lethal, but cell viable. The trc mutation has been used as a cuticular marker in genetic screens. The Trc phenotype of genetic mosaic flies shows splitting, clustering, or branching of polarised structures, like epidermal hairs decorating the adult cuticular surface, the shafts of bristle sense organs, the lateral extensions of the arista, and the larval denticle (Geng et al, 2000). The morphology and polarity of these structures is, however, normal except for the region of the branch point. This is different from mutations in the frizzled tissue polarity pathway such as inturned or actin cytoskeleton components such as crinkled, singed, or forked which exhibit an altered polarity of cellular projections or abnormal morphology all regions of the structure (Adler and Lee, 2001). But Trc might interact with the actin cytoskeleton, because treatment of differentiating cells by cytochalasin D or latrunculin A, inhibitors of actin polymerisation,

phenocopied the branched hairs and bristles found in trc mutant clones. A weak trc mutant was hypersensitive to cytochalasin D treatment (Geng et al, 2000). But the treatment with these drugs also results in short, fat and occasionally malformed hairs and bristles, which does not occur in trc mutants. These differences indicate that TRC is not simply required for actin polymerisation; it is more likely that TRC interacts with the actin cytoskeleton in a more subtle way. In drosophila sensory neurons, TRC is important for the control of dendritic branching and tiling (tiling refers to the complete but nonredundant coverage of a receptive field by dendrites of functionally homologous neurons). Dendrites of trc mutants display excessive terminal branching and fail to avoid homologous dendritic branches, resulting in significant overlap of the dendritic fields (Emoto et al, 2004).

8.2.2. C. elegans

The C. elegans gene sax-1 encodes a protein kinase with 66% identity and 74% similarity to human NDR1. Sax-1 mutants are organismal viable, but they possess defects in neuronal cell shape and polarity. Certain types of neuronal cells of sax-1 mutants have expanded to irregular cell bodies and initiate ectopic neurites. This suggests that SAX-1 functions to restrict cell and neurite growth (Zallen et al, 2000). SAX-1 kinase is important for mechanosensory neurite termination and tiling. SAX-1 functions in a pathway with SAX-2, a Furry like protein. During C. elegans development, the posterior PLM mechanosensory dendrite overlaps with the anterior ALM mechanosensory neuron. The PLM process growth slows down during a discrete period of time, between an early period of rapid outgrowth and a later period of maintained growth. Thereby the overlap is eliminated. In sax-2 mutants, anterior and posterior mechanosensory processes overlap, because the

growth of the PLM sensory dendrite does not slow down between the active growth and maintenance growth phases (Gallegos and Bargmann, 2004).

8.2.3. Function of NDR kinases in mammalians

New results from our lab point to a role for NDR kinase in the control of the cell cycle and cell division: NDR activity and phosphorylation of its hydrophobic motif site Thr-444 is tightly cell cycle regulated. Activity and Thr-444 phosphorylation increases at the G1/S boundary and persists until mitosis and cytokinesis. Mouse embryonic fibroblasts derived from NDR1 knockout mice display severe proliferation defects, including delayed entry into S-phase accompanied by reduced expression of cyclin A, and decelerated mitosis. Elimination of NDR2 in mouse embryonic fibroblasts from NDR1 knockout mice leads to an exacerbation of the proliferation blocks, extensive cell death and to accumulation of polyploid and multinucleated cells, which is due to cytokinesis defects. (R. Tamaskovic et al, manuscripts in preparation.).

Furthermore, it was shown that hNDR2 mRNA is induced in the mouse amygdala during fear memory consolidation. Transfection of hNDR2 in PC12 cells leads to decreased cell spreading and changes in neurite outgrowth (Stork et al, 2004).

8.2.4 Role of NDR kinase in disease

Members of the NDR kinase family are involved in the regulation of the cell cycle and cell morphology. Therefore, human NDR kinase may be involved in control of cell proliferation and cancer. The Ndr1 as well as the Ndr2 gene location 6p21

and 12p11 respectively belong to regions that were described as cancer amplicons (Manning et al, 2002). NDR1 mRNA is consistently upregulated in ductal carcinoma in situ with intraductal necrosis ($DCIS^{necrosis+}$) compared to DCIS without intraductal necrosis. $DCIS^{necrosis+}$ cases have a higher risk of recurrence and/or the progression of DCIS to invasive tumors (Adeyinka et al, 2002). NDR1 kinase was found to be hyperactivated in some S100B positive melanoma cell lines (Millward et al, 1998). S100B activates NDR in vitro (Tamaskovic et al, 2003), and S100B serum levels are used as a prognostic marker for tumors with poor prognosis (Hauschild et al, 1999). Human NDR2 is up regulated in the highly metastatic non-small cell lung cancer cell line NCI-H460 (Ross et al, 2000). Taken together, NDR kinases are involved in a signalling pathway that controls cell proliferation and morphology, and its deregulation may be involved in the pathogenesis of some malignant disorders.

Furthermore, NDR1 and NDR2 kinases were shown to be incorporated into HIV-1 particles. NDR1 and NDR2 were cleaved by the HIV-1 protease both within the virions and within the producer cells, and these truncation inhibited NDR kinase activity. (Devroe et al, 2005).

9. Conclusions and future prospects

NDR kinase is highly conserved during evolution. NDR kinase is part of a novel conserved signalling pathway, which is implicated in the control of cell morphology and the cell cycle. The signalling pathway involves MOB proteins. The results presented in this thesis describe a new mechanism of kinase regulation by MOB proteins. Binding of MOB to the SMA (S100 and MOB association)-domain of NDR induces the release of the autoinhibition that is caused by the autoinhibitory sequence (AIS) and thereby stimulates NDR kinase activity.

In the future, the identity and function of other components of the NDR signalling pathway will be unravelled. For example, the kinase that phosphorylates the hydrophobic motif site has been identified as the Ste-20 like kinase MST3 (Stegert et al, manuscript in preparation). The function of other proteins which are orthologues of components of the yeast RAM-signalling network, like the human Furry-like protein, need to be investigated.

NDR family kinases are involved in the regulation of cell morphology and the cell cycle. The most important future task will be to unravel the function of mammalian NDR1 and NDR2 kinase. New results in our lab show that NDR is cell cycle regulated and seem to play a role in cell cycle progression. Further work will be required to investigate the exact role of NDR in the cell cycle. The physiological role of NDR is also investigated by the use of NDR1 and NDR2 knockout mice. There was no phenotype detected so far for the NDR1 single knockout mice, therefore it is likely that the two isoforms compensate each other. The NDR2 and the double knockout will help to shed light on the physiological function of NDR kinase.

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11.Curriculum Vitae

Name: Bichsel
Vorname: Samuel
Adresse Im Surinam 91, 4058 Basel
Geburtsdatum: 5.5.1970
Geburtsort: Grenchen
Heimatort: Hasle BE

Berufstätigkeit:
11/00-02/05 Doktorarbeit, FMI, Gruppe Dr. Brian A. Hemmings
08/96-10/96 Temporäranstellung, Siegfried AG, Zofingen
04/95-09/95 Pharmabiologielaborant, Ciba, 75%
04/91-04/95 Pharmabiologielaborant, Ciba, 100%
04/88-04/91 Berufslehre Pharmabiologielaborant, Ciba-Geigy, Basel

Studium:
11/00-04/05 Doktoratsstudium, Biochemie, Basel (Dr. Brian A. Hemmings, Prof. Tom Bickle, Prof. Patrick Matthias)
10/96-11/00 Studium Biologie 2, Basel

Schulbildung:
11/91-2/96 Maturavorbereitung AKAD Zürich (Fernunterricht)
04/88-04/91 Gewerbeschule Basel
04/84-04/88 Bezirksschule Grenchen
04/78-04/84 Primarschule Grenchen

Abschlüsse:
2005: Doktorat Biochemie
2000: Diplom, Biologie 2
1996: eidg. Matur, Typus C
1991: eidg. Fähigkeitszeugnis Pharmabiologielaborant

Publications:

Bichsel, S.J., Tamaskovic, R., Stegert, M.R. and Hemmings, B.A. (2004) Mechanism of activation of NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. *J Biol Chem*, **279**, 35228-35235.

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