Calcium regulation of Ndr protein kinase mediated by S100 calcium-binding proteins

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Ndr is a nuclear serine/threonine protein kinase that belongs to a subfamily of kinases identified as being critical for the regulation of cell division and cell morphology. The regulatory mechanisms that control Ndr activity have not been characterized previously. In this paper, we present evidence that Ndr is regulated by EF-hand calcium-binding proteins of the S100 family, in response to changes in the intracellular calcium concentration. *In vitro*, S100B binds directly to and activates Ndr in a Ca^{2+} -dependent manner. Moreover, Ndr is recovered from cell lysates in anti-S100B immunoprecipitates. The region of Ndr responsible for interaction with Ca^{2+} /S100B is a basic/hydrophobic motif within the N-terminal regulatory domain of Ndr, and activation of Ndr by Ca^{2+} /S100B is inhibited by a synthetic peptide derived from this region. In cultured cells, Ndr is rapidly activated following treatment with Ca^{2+} ionophore, and this activation is dependent upon the identified Ca^{2+} /S100B-binding domain. Finally, Ndr activity is inhibited by W-7 in melanoma cells overexpressing S100B, but is unaffected by W-7 in melanoma cells that lack S100B. These results suggest that Ndr is regulated at least in part by changes in the intracellular calcium concentration, through binding of S100 proteins to its N-terminal regulatory domain.

Keywords: calcium signalling/Ndr protein kinase/
nucleus/protein phosphorylation/S100 proteins

Introduction

Ndr is a conserved and widely expressed nuclear serine/threonine protein kinase that was recently identified in cDNA libraries derived from human fetal brain, *Drosophila* and *Caenorhabditis elegans* (Millward *et al.*, 1995). Although the cellular substrates of Ndr have not yet been identified, Ndr is structurally related to several protein kinases that regulate cell division and cell morphogenesis, suggesting that it too might perform a similar function. This recently identified subfamily of kinases comprises, in addition to Ndr, the budding yeast kinase Dbf2, fission yeast Orb6, *Drosophila* Wts, *Neurospora* Cot-1, the human myotonic dystrophy kinase DMPK and the mammalian Rho-associated kinase (also referred to as p160^{ROCK} or

ROK α). Dbf2 is a cell-cycle-regulated kinase whose activity is required for progression through anaphase; at the restrictive temperature, yeast cells carrying temperature-sensitive alleles of Dbf2 arrest with a uniform large-budded 'dumb-bell' morphology resulting from a failure to complete cell division (Johnston *et al.*, 1990; Toyn and Johnston, 1994). Wts (also known as LATS) has been identified as a tumor suppressor gene in *Drosophila*: Wts^{-/-} cells overproliferate compared with surrounding heterozygous cells, are rounded and hypertrophic, and display abnormal extracellular matrix metabolism (Justice *et al.*, 1995; Xu *et al.*, 1995). Likewise, Orb6 coordinates the control of cell proliferation with cell morphogenesis in fission yeast (Verde *et al.*, 1998). The Rho-associated kinase regulates cytoskeletal structure and its *C.elegans* homologue, LET-502, has been shown to be essential for changes in cell shape that occur during early embryogenesis (Leung *et al.*, 1996; Wissmann *et al.*, 1997). The cot-1 gene regulates hyphal growth and branching in *Neurospora crassa* (Yarden *et al.*, 1992). Finally, human DMPK is abnormally expressed in myotonic dystrophy patients due to the expansion of a trinucleotide repeat in its 3' untranslated region (Brook *et al.*, 1992; Fu *et al.*, 1993). These kinases (including Ndr) share 40–60% amino acid identity with each other in their catalytic domains and also contain conserved regions in their putative regulatory domains. The structural similarity of these kinases suggests that they might have common substrates and related cellular functions.

Within the protein kinase superfamily, the subgroup containing Ndr, Dbf2, Cot-1, Wts, Orb6, Rho kinase and DMPK is most closely related to the 'AGC' group of serine/threonine kinases, which includes cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C and protein kinase B (Hanks and Hunter, 1995). Each of these kinases is regulated by one or more second messengers (cAMP, cGMP, Ca^{2+} /diacylglycerol or phosphoinositides, respectively). Ca^{2+} is perhaps the most pleiotropic of all known second messengers. Transient elevations in the intracellular Ca^{2+} concentration are implicated in the regulation of many cellular functions, including (to name but a few) cell growth and division, cell motility, transcription, protein synthesis and apoptosis (Taylor *et al.*, 1989; Brostrom and Brostrom, 1990; Kao *et al.*, 1990; Werlen *et al.*, 1993; Lam *et al.*, 1994). One of the reasons why this diversity of signalling is possible is that Ca^{2+} binds to multiple classes of Ca^{2+} -binding proteins, each of which can, in turn, regulate multiple downstream effectors. Among the major intracellular Ca^{2+} receptors are calmodulin (CaM) and S100 proteins. CaM and S100 proteins are members of a large family of 'EF hand' Ca^{2+} -binding proteins which share conserved structural features and a common mechanism of action (Kretsinger *et al.*, 1991). These proteins bind Ca^{2+} through

Fig. 1. Identification of Ndr substrate peptides. Peptides, listed on the left, were tested for phosphorylation by GST-Ndr WT as described in Materials and methods. The extent of phosphorylation of each peptide is expressed as a percentage relative to the optimal substrate peptide (KKRNRRLSVA). In each peptide, amino acid substitutions relative to the optimal substrate peptide are underlined. The serine phosphoacceptor (position 0) is shown in bold.

their EF-hand motifs and undergo a Ca^{2+} -induced conformational change to expose a hydrophobic effector protein-binding surface (Zhang *et al.*, 1995; Smith *et al.*, 1996), which in turn allows them to bind to and regulate an array of secondary effector proteins. In this paper we present evidence that the protein kinase activity of Ndr is regulated by the second messenger Ca^{2+} , and that this regulation is brought about through calcium-dependent interaction of Ndr with members of the S100 family of EF-hand calcium-binding proteins. *In vitro*, Ndr interacts with S100 proteins in a Ca^{2+} -dependent manner, and certain isoforms of the S100 protein family can confer Ca^{2+} sensitivity on Ndr protein kinase activity. S100 proteins are known to be involved in the regulation of cell growth and cell morphology, and Ndr may represent one S100 effector protein important for these functions.

Results

Development of an Ndr protein kinase assay

When incubated *in vitro* with Mg-ATP, Ndr undergoes intramolecular serine/threonine autophosphorylation, but fails to transphosphorylate substrates typically used in kinase assays such as histone, myelin basic protein, casein and phosvitin (Millward *et al.*, 1995). To develop a convenient *in vitro* kinase assay for Ndr, an assembled library of ~50 synthetic peptides was screened for potential Ndr substrates. Several peptides were identified which acted as Ndr substrates, and a selection of these are shown in Figure 1. Of the peptides tested, the best substrate for Ndr had the sequence KKRNRRLSVA. By comparing the phosphorylation of this peptide with other closely related peptides, several conclusions regarding the substrate specificity of Ndr could be drawn. Replacement of the basic amino acids at positions -6, -5, -3 or -2 (where position 0 is the serine phosphoacceptor) with non-polar residues was strongly inhibitory; double substitution of the -7 and -6 positions with alanine completely abolished phosphorylation by Ndr. Substitution of valine for proline at the +1 position was also inhibitory. Finally, Ndr showed a preference for serine over threonine as the phosphoacceptor. Based on this information, our current

Fig. 2. Ca^{2+} -dependent interaction of Ndr protein kinase with CaM and S100B. (A) Purified GST (top panels) or GST-Ndr (lower panels) was tested for binding to CaM-agarose. Binding and washing was carried out in a buffer supplemented with either CaCl_2 or EGTA, as indicated below the panels. After the binding assay, equal portions (20%) of the input material (Input), the binding supernatant (Unbound) and the EGTA eluate (Eluate) were immunoblotted with an anti-GST antiserum (top panels) or with Ab⁴⁵²⁻⁴⁶⁵ (lower panels). (B) Binding of GST (top panels) and GST-Ndr (lower panels) to S100B-agarose were tested as in (A).

best estimate of the optimal Ndr substrate sequence is $\text{R/K}^{\text{R/K}} \text{R/K}^{\text{R/K}} \text{R/K}^{\text{R/K}} \text{S n x}$, where x is any amino acid and n is any amino acid except proline. Notably, however, the K_m of glutathione S-transferase (GST)-Ndr for this substrate was only ~500 μM (data not shown), suggesting that better Ndr substrates exist. In subsequent experiments, the peptide KKRNRRLSVA was used as an Ndr substrate.

Ca^{2+} -dependent binding of Ndr protein kinase to CaM and S100 proteins

During initial experiments aimed at characterizing the chromatographic behaviour of Ndr, we observed that Ndr could be partially purified from mammalian cell extracts using CaM-agarose (data not shown). *In vitro* binding assays showed that this interaction was direct: when purified GST-Ndr was incubated with CaM-agarose and Ca^{2+} , ~50% of the added GST-Ndr bound to the resin and, after washing, was eluted by addition of EGTA (Figure 2A). Binding was not detectable in the absence of free Ca^{2+} . Native GST did not interact with CaM-agarose either in the presence or absence of Ca^{2+} .

We also analysed binding of GST-Ndr to S100B-agarose, since the binding properties of CaM and S100 proteins often overlap (see Discussion). GST-Ndr, but not GST, bound in a Ca^{2+} -dependent manner to S100B-agarose (Figure 2B). Under conditions identical to those used in Figure 2A, Ca^{2+} /S100B-agarose completely removed GST-Ndr from solution, whereas Ca^{2+} /CaM-agarose bound only ~50% of the available GST-Ndr. This

Fig. 3. Ca²⁺-dependent activation of Ndr by the CaM-related proteins S100B and S100A1. (A) GST-Ndr peptide kinase activity was measured without further additions or in the presence of 0.1 mM CaCl₂ and bovine CaM, recombinant human S100B, or recombinant human S100A1, as indicated. Either wild-type (Ndr WT) or kinase-negative (Ndr K118A) GST-Ndr was used. Bars represent mean $\pm$ standard deviation of duplicate assays. (B) 1 μ g of GST-Ndr (in solution; $\sim$ 0.5 μ M) was assayed in the presence of various concentrations (0–10 μ M) of bovine S100B homodimer and either 0.1 mM CaCl₂ (circles) or 2 mM EGTA (diamonds). Points represent mean $\pm$ standard deviation of triplicate determinations. (C) One microgram of GST-Ndr was assayed for peptide kinase activity at various concentrations (0–500 μ M) of CaCl₂. To obtain '0 μ M Ca²⁺' the assay was supplemented with 2 mM EGTA. Assays contained either GST-Ndr alone (diamonds) or GST-Ndr together with 10 μ M bovine S100B homodimer (circles). Points represent mean $\pm$ standard deviation of triplicate determinations.

suggests that Ndr binds with higher affinity to Ca²⁺/S100B than to Ca²⁺/CaM. It was also apparent that a certain proportion of the GST-Ndr input protein was removed from solution by S100B-agarose in the absence of free Ca²⁺, consistent with the idea that binding of Ndr to S100B is also possible in the absence of Ca²⁺, albeit with considerably lower affinity.

Activation of Ndr protein kinase by S100 proteins

The Ca²⁺-dependent binding of Ndr to CaM and S100 proteins prompted us to test whether any of these proteins modulate Ndr protein kinase activity. Under conditions that would effectively activate known CaM-dependent kinases, Ca²⁺/CaM had only a minor effect (<2-fold activation) on Ndr peptide kinase activity (Figure 3A), and had no effect on Ndr autophosphorylation (data not shown). However, inclusion of Ca²⁺/S100B or the closely related Ca²⁺/S100A1 led to a significant activation of Ndr peptide kinase activity (Figure 3A). This activation was isoform specific; in identical assays, neither S100A2 (originally called S100L), S100A4 (also known as CAPL or mts1) nor S100A6 (calcylin) caused any activation of Ndr (data not shown). The apparent activation of Ndr in the presence of Ca²⁺/S100B and Ca²⁺/S100A1 was not due to a kinase activity contaminating the S100 proteins, because no peptide kinase activity was recorded when kinase-negative GST-Ndr (containing a Lys¹¹⁸→Ala mutation in the ATP binding pocket) was used in place of wild-type GST-Ndr (Figure 3A).

Since Ca²⁺/S100B was found to be the strongest activator of Ndr, we focused subsequent experiments on Ndr activation by this protein. In order to estimate the binding affinity of S100B for Ndr, kinase assays were performed in which GST-Ndr was mixed with various concentrations of purified bovine S100B homodimer, either in the presence or absence of Ca²⁺ (Figure 3B). The maximal activation of Ndr by Ca²⁺/S100B was $\sim$ 7-fold, and the concentration of Ca²⁺/S100B required for half-maximal

activation (EC₅₀) was $\sim$ 0.5 μ M. Apo-S100B (assayed in the presence of 2 mM EGTA) caused a limited activation of Ndr, but maximal activation was lower, and the EC₅₀ was substantially higher ($\sim$ 5 μ M). To investigate further the Ca²⁺ dependence of Ndr activation by S100B, Ndr activity was assayed with or without added S100B over a range of Ca²⁺ concentrations. Ndr in its monomeric form showed no Ca²⁺ sensitivity (Figure 3C); however, in the presence of 10 μ M S100B, Ndr activity became sensitive to changes in the Ca²⁺ concentration. This Ca²⁺ dependence was most acute at Ca²⁺ concentrations within the range 1–100 μ M, in agreement with previous determinations of the *in vitro* binding affinity of S100B for Ca²⁺ (Baudier *et al.*, 1986). As noted above, S100B also caused a limited activation of Ndr in the absence of added Ca²⁺.

Mechanism of activation of Ndr by Ca²⁺/S100B

In addition to increasing the peptide kinase (i.e. transphosphorylating) activity of Ndr, Ca²⁺/S100B also stimulated Ndr autophosphorylation (Figure 4A), which occurs by an intramolecular mechanism (Millward *et al.*, 1995). No autophosphorylation was observed using kinase-negative GST-Ndr. Since several known protein kinases are activated by autophosphorylation, we tested whether this Ca²⁺/S100B-induced autophosphorylation of Ndr might underlie its activation in response to Ca²⁺/S100B. In the experiment shown in Figure 4B, GST-Ndr was immobilized on glutathione-agarose beads and preincubated with or without Ca²⁺/S100B, in the presence or absence of Mg-ATP (either to allow or prevent autophosphorylation). The beads were then washed and assayed for Ndr peptide kinase activity using radiolabelled ATP, again in the presence or absence of Ca²⁺/S100B. Preincubation of GST-Ndr with Mg-ATP alone increased both its autonomous (Ca²⁺/S100B-independent) and Ca²⁺/S100B-stimulated peptide kinase activities, showing that autophosphorylation stimulates Ndr activity towards

Fig. 4. Mechanism of activation of Ndr by Ca^{2+} /S100B. (A) GST-Ndr K118A or GST-Ndr WT (1 μg of protein bound to 2 μl glutathione-agarose beads) was incubated in kinase buffer containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 0.1 mM Ca^{2+} , in the presence or absence of 10 μM bovine S100B, as indicated. The beads were then washed, boiled in sample buffer, and analysed by SDS-PAGE and autoradiography. (B) GST-Ndr WT (1 μg of protein bound to 2 μl glutathione-agarose beads) was preincubated in kinase buffer containing 0.1 mM CaCl_2 , with or without 5 mM MgCl_2 /100 μM unlabelled ATP, and with or without 10 μM bovine S100B, as indicated below the graph. After 1 h at 30°C , the beads were washed with buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 0.5 mM EGTA) to remove S100B and Mg-ATP. Following this, the beads were washed in kinase buffer and assayed for peptide kinase activity either in the presence (filled bars) or absence (open bars) of 10 μM Ca^{2+} /S100B. Bars represent mean $\pm$ standard deviation of duplicate determinations. (C) Model depicting the mechanism of activation of Ndr by Ca^{2+} /S100B. See text for details.

exogenous substrates. Preincubation of GST-Ndr with both Mg-ATP and Ca^{2+} /S100B resulted in an even greater autoactivation of the peptide kinase activity, consistent with the finding that Ca^{2+} /S100B stimulates Ndr autophosphorylation (Figure 4A). Preincubation with Ca^{2+} /S100B alone had no effect, confirming that activation by Ca^{2+} /S100B in the preincubation step is mediated by Ndr autophosphorylation.

Ca^{2+} /S100B increased the rate of Ndr autophosphorylation by ~ 2.5 -fold (as estimated by phosphorimager analysis of Figure 4A), and it stimulated autophosphorylation-dependent Ndr activation by ~ 2 -fold (compare the third and fifth columns or the fourth and sixth columns in Figure 4B). However, the total activation of Ndr by Ca^{2+} /S100B was considerably larger than this (on average ~ 7 -fold; compare open bars with filled bars). Thus, the increased autophosphorylation of Ndr caused by Ca^{2+} /S100B only partially accounts for the activation of Ndr.

Fig. 5. Identification of the CaM/S100 binding domain of Ndr protein kinase. (A) Schematic diagram of Ndr protein kinase showing the kinase catalytic domain and a basic/hydrophobic sequence in the N-terminal regulatory domain. Numbers refer to the amino acid sequence of human Ndr. (B) Lysates from COS-1 cells expressing wild-type Ndr (WT) or N-terminal deletion mutants ($\Delta 65-81$, $\Delta 1-84$) were supplemented with Ca^{2+} and mixed for 2 h with CaM-agarose. From each binding assay, equal portions (60%) of the unbound fraction (Unbound) and the EGTA-eluted proteins (Eluate) were immunoblotted with Ab⁴⁵²⁻⁴⁶⁵. (C) GST-Ndr WT was assayed for peptide kinase activity in the absence (diamonds) or presence (circles) of 10 μM Ca^{2+} /S100B. A synthetic peptide corresponding to amino acids 62–86 of human Ndr (KRLRRSAHARKETEFRLRLKRTLGL) was added at various concentrations (10 nM–100 μM), as indicated. Points represent mean $\pm$ standard deviation of duplicate measurements.

A likely explanation for this is that, in addition, Ca^{2+} /S100B stimulates Ndr kinase activity directly, in an autophosphorylation-independent manner. This suggests a dual mechanism for activation of Ndr by Ca^{2+} /S100B: Ca^{2+} /S100B induces Ndr autophosphorylation (which in turn stimulates Ndr transphosphorylation activity) but, in addition, there is also an allosteric stimulation of Ndr transphosphorylation activity that results directly from the protein–protein interaction itself (Figure 4C).

Identification of the CaM/S100 binding domain of Ndr protein kinase

Inspection of the amino acid sequence of Ndr revealed a region, amino acids 62–86 in the N-terminal regulatory domain, which is enriched in basic and hydrophobic amino acids, and which is predicted (on the basis of amino acid content) to be α -helical (Figure 5A). Such characteristics are typical of CaM and S100 binding sequences (see Discussion). To test whether this region might be responsible for the interaction of Ndr with CaM and S100 proteins, deletion mutants of Ndr were expressed in COS-1 cells and assayed for binding to CaM-agarose. Two mutants were tested, lacking either the complete N-terminal domain of Ndr ($\Delta 1-84$) or a 17-amino-acid region within the

putative CaM/S100-binding sequence ($\Delta 65$ –81). Wild-type Ndr was efficiently removed from the lysate by CaM–agarose, as assessed by Western blotting (Figure 5B, top panel). Deletion of amino acids 65–81 caused a large reduction in CaM binding, to ~10% of the level of wild-type Ndr (Figure 5B, middle panel). When the entire N-terminal domain of Ndr was removed, binding to CaM–agarose was no longer detectable (Figure 5B, lower panel).

In order to determine whether this same region is also responsible for interaction with Ca²⁺/S100B, a peptide corresponding to amino acids 62–84 of human Ndr was synthesized and added to kinase assays containing either GST–Ndr alone or GST–Ndr together with Ca²⁺/S100B. At concentrations of ≤ 100 μ M, this peptide had no significant inhibitory effect on the activity of monomeric Ndr (Figure 5C). However, the same peptide caused a dose-dependent inhibition of Ca²⁺/S100B-stimulated Ndr activity. This inhibition was detectable at peptide concentrations > 1 μ M, and was essentially complete at 100 μ M peptide. This antagonism of the interaction between Ndr and Ca²⁺/S100B confirms that the CaM/S100-binding domain of Ndr overlaps with, or is contained within, amino acids 62–86.

Regulation of Ndr by Ca²⁺/S100B in mammalian cells

To investigate whether Ndr and S100B are capable of forming complexes within the cell, COS-1 cells were cotransfected with expression vectors encoding Ndr and S100B, and complex formation assayed by co-immunoprecipitation. When cells transfected with both vectors were lysed and immunoprecipitated with anti-S100B antiserum, Ndr immunoreactivity was detected in the immunoprecipitate (Figure 6A). Ndr was not detected in anti-S100B immunoprecipitates from cells that were untransfected or cells that were transfected with S100B alone; however, a small amount of Ndr was immunoprecipitated by anti-S100B from cells transfected with Ndr alone, which suggests that overexpressed Ndr complexes with either endogenous S100B or a protein antigenically related to S100B. The co-immunoprecipitation of Ndr with S100B was specific, because it was abolished by deletion of the N-terminus of Ndr (containing the previously identified CaM/S100 binding domain).

We next attempted to study the role of Ca²⁺ and S100B in the regulation of Ndr kinase activity *in vivo*. In these experiments, cells were transfected with haemagglutinin-tagged Ndr (HA–Ndr) and Ndr peptide kinase activity measured in anti-HA immune complexes following cell lysis. To preserve complexes of Ndr and S100B during immunoprecipitation, it had been necessary to prepare non-detergent cell lysates (Figure 6A). However, under these conditions, anti-HA immunoprecipitates contained an unacceptably high background of non-specific kinase activity. Therefore, HA–Ndr was immunoprecipitated as a monomer (i.e. from lysates containing detergent and EGTA); if Ca²⁺/S100B stimulates Ndr autophosphorylation, it should be possible to detect an activation of monomeric Ndr in an *in vitro* kinase assay following binding of Ca²⁺/S100B to Ndr within the cell. Note, however, that since this assay measures only the activation of Ndr resulting from Ca²⁺/S100B-induced autophos-

Fig. 6. Functional association of Ndr with Ca²⁺/S100B in mammalian cells. (A) COS-1 cells were transfected with expression plasmids for Ndr WT, Ndr $\Delta 1$ –84 and S100B, as indicated. The DNA concentration during transfection was kept at 2 μ g/ml using empty vector DNA (pECE). Seventy-two hours later, non-detergent cell lysates were prepared and analysed for Ndr expression (top panel), S100B expression (middle panel) and association of Ndr with anti-S100B immunoprecipitates (lower panel). (B) COS-1 cells were cotransfected with pECE–S100B and either pECE–HA–Ndr–WT (circles) or pECE–HA–Ndr– $\Delta 65$ –81 (diamonds), and, after 72 h, were treated with 20 μ M A23187 for various lengths of time. Detergent lysates were then prepared and immunoprecipitated with 12CA5, and the immune complexes assayed for Ndr peptide kinase activity. Points represent mean $\pm$ standard deviation of duplicate immunoprecipitations. Treatment of cells with solvent alone (0.1% DMSO) did not affect Ndr activity (data not shown). Ten microgrammes of each extract were immunoblotted with 12CA5 to confirm equal expression of each construct at each time point (lower panels).

phorylation, it would tend to underestimate the true extent of Ndr activation (Figure 4C).

The activity of HA–Ndr in COS-1 cells cotransfected with S100B was only slightly higher (~1.3-fold) than when the cells were transfected with HA–Ndr alone (data not shown); this argues that COS-1 cells already contain sufficient S100B, or a related protein, to activate Ndr. Possibly, COS-1 cells express S100A1, since S100A1 is very abundant in kidney (Kato *et al.*, 1986). Notably, a mutant form of Ndr (HA–Ndr– $\Delta 65$ –81), containing a deletion in the S100-binding domain, showed a markedly reduced basal activity (5–10% of wild-type Ndr activity) suggesting that Ca²⁺ and S100 binding play a major role in generating catalytically active Ndr (Figure 6B and data not shown). Cells cotransfected with HA–Ndr (WT or

Fig. 7. Evidence that S100B overexpression can cause hyperactivation of Ndr in melanoma cells. (A) Immunodetection of endogenous Ndr. Extracts from COS-1 cells transfected with pECE-Ndr (20 μ g protein; lane 1), pECE (20 μ g; lane 2) and a HeLa cell extract (400 μ g; lane 3) were subjected to immunoprecipitation with anti-Ndr antibodies; the immunoprecipitates were separated by SDS-PAGE and blotted with biotinylated Ab¹⁻⁴⁶⁵ and streptavidin-peroxidase. Molecular weight markers (kDa) are indicated on the right. (B) Expression of S100B (upper panels) and Ndr (lower panels) in HeLa cells, A375 melanoma cells and M960618 melanoma cells, as detected by immunoprecipitation/immunoblotting. (C) Effect of W-7 on Ndr activity in S100B-negative (A375; open bars) and S100B-positive (M960618; closed bars) human melanoma cells. Cells ($\sim 3 \times 10^6$ /10 cm dish) were left untreated or were treated with 50 μ M W-7 for 75 min. Extracts were prepared and Ndr activity measured by immune complex kinase assay. Bars represent mean $\pm$ standard deviation of duplicate immunoprecipitations.

$\Delta 65-81$) and S100B were then treated for various lengths of time with the calcium ionophore A23187. Addition of ionophore to cells caused a rapid activation of wild-type HA-Ndr, which peaked after 5 min and declined thereafter (Figure 6B). Importantly, the $\Delta 65-81$ Ndr mutant, defective in binding Ca^{2+} /S100B, was completely unresponsive to increased intracellular Ca^{2+} . This suggests that the wild-type HA-Ndr had indeed become activated as a result of Ca^{2+} -induced autophosphorylation, caused by binding of S100B to its N-terminal regulatory domain. At the peak of activation, the activity of Ndr was increased 1.7-fold above the basal level; however, as discussed above, this assay is expected to underestimate the true degree of activation. Western blot analysis confirmed that HA-Ndr-WT and HA-Ndr- $\Delta 65-81$ were expressed at the same level, and that expression levels were unaffected by ionophore treatment (Figure 6B). Ndr was also activated by ionophore treatment in cells that had not been transfected with S100B (data not shown), again suggesting that the transfected Ndr can associate with endogenous S100B or a related S100 protein.

S100B and Ndr in melanoma cells

In order to study the functional relationship between Ndr and S100B in untransfected cells, a polyclonal antiserum was raised using GST-Ndr as immunogen. The specificity of the purified antibodies was tested by immunoprecipitation followed by Western blotting of the immunoprecipitates. HeLa cell lysates contained an endogenous 55 kDa immunoreactive protein that co-migrated with the product of the cloned Ndr cDNA produced in transfected COS-1 cells (Figure 7A). In addition, a band of 53 kDa was detected, which could be either a proteolytic fragment of Ndr or a closely related isoform. The identity of these bands was further confirmed by preincubation of the blotting antibody with antigen: both bands disappeared

following preincubation with GST-Ndr, but not following preincubation with GST (data not shown).

S100B is highly overexpressed in the majority of melanomas (Cocchia *et al.*, 1981; Marks *et al.*, 1990; Boni *et al.*, 1997), to the extent that antibodies against S100A1/S100B are widely used for tumour typing and the diagnosis of melanoma (Cochran *et al.*, 1993). Since Ndr is activated by Ca^{2+} /S100B, overexpression of S100B might result in hyperactivation of Ndr in melanoma cells. To investigate this possibility, a panel of melanoma cell lines was analysed for S100B expression, Ndr expression and Ndr peptide kinase activity; Ndr kinase activity was measured in an immune complex kinase assay, using the antibody described above. Of nine melanoma cell lines analysed, seven expressed S100B, while all nine expressed Ndr (data not shown). In most of these cell lines, Ndr kinase activity was in the range 0.01–0.03 mU/mg extract, but one of the S100B-positive melanomas (M960618) showed Ndr activity that was 5–10 times higher than in the other cell lines (data not shown). We further analysed this particular cell line, and compared it to one of the melanoma cell lines lacking S100B (A375). As mentioned above, Ndr activity was higher in M960618 cells than in A375 cells (Figure 7C), correlating with the expression of S100B (Figure 7B). To determine whether S100B overexpression is the cause of the elevated Ndr kinase activity found in M960618 cells, we made use of the compound W-7. W-7 was originally identified as a cell-permeable inhibitor of CaM, which binds to CaM in a Ca^{2+} -dependent manner and antagonizes the interaction of CaM with its target proteins (Hidaka *et al.*, 1981; Hidaka and Tanaka, 1983); however, W-7 also binds to several S100 proteins, including S100B, in a Ca^{2+} -dependent manner (Hidaka *et al.*, 1983; Umekawa *et al.*, 1983) and thus would be expected to antagonize S100 proteins in the same way as CaM. Treatment of S100B-

negative A375 cells with W-7 did not affect Ndr activity (Figure 7C). In contrast, the same treatment in M960618 cells reduced Ndr activity by ~80%, to a level similar to that present in A375 cells. These results suggest that S100B overexpression is indeed the cause of Ndr hyperactivation in M960618 cells.

Discussion

In this paper we have presented data which suggest that Ndr, a nuclear serine/threonine protein kinase, is regulated by changes in the intracellular calcium concentration through direct association with EF-hand calcium-binding proteins of the S100 family. Initially, Ca²⁺/CaM was found to bind Ndr, without affecting its kinase activity; subsequently we found that Ca²⁺/S100B bound more avidly to Ndr, and also stimulated its kinase activity. This activation was specific for the S100B and, to a lesser extent, the S100A1 isoforms. The other S100 proteins tested, which share 40–60% amino acid identity with S100A1 and S100B (Schäfer *et al.*, 1995), had no effect on Ndr kinase activity.

The overlapping specificity of CaM and S100 proteins for interaction with target proteins and synthetic drugs has been noted several times before (Marshak *et al.*, 1981; Hidaka *et al.*, 1983; Baudier *et al.*, 1987; Ivanenkov *et al.*, 1995). It has also been observed previously that CaM and S100 proteins can have differential effects on a common target protein, even when they bind to the same site: for example, twitchin kinase binds CaM and S100A1, but is only activated by the latter (Heierhorst *et al.*, 1997); conversely, CaM kinase II can also bind S100B, but is only activated by CaM (Baudier *et al.*, 1995). In a number of cases, the domains of these proteins responsible for binding to CaM or S100 proteins have been identified. While no exact consensus exists between the various binding domains, several conserved features can be identified: typically, they are short linear epitopes, contain an unusually high proportion of basic and hydrophobic amino acids, and in certain cases have the potential to form an amphipathic α -helix (O'Neil and DeGrado, 1990; James *et al.*, 1995). These features are present in the identified CaM/S100 protein-binding domain of Ndr.

The mechanism of activation of Ndr by Ca²⁺/S100B shows certain parallels to the way in which CaM-dependent kinases are activated by Ca²⁺/CaM: binding of the activator to the kinase stimulates autophosphorylation of the kinase, which then in turn stimulates both the autonomous (Ca²⁺-independent) and Ca²⁺-stimulated activities. Thus a transient, non-covalent protein–protein interaction causes a stimulatory covalent modification (phosphorylation) of the kinase, which remains in place until the kinase is dephosphorylated by the appropriate phosphatase. However, a major difference between Ndr and most of the known CaM- or S100-dependent kinases is the location of the CaM/S100 binding domain of Ndr. In the classical CaM-dependent kinases (and also in the twitchin kinase), the CaM/S100 binding domain is located directly C-terminal to the kinase catalytic domain, and it overlaps with (or is identical to) an autoinhibitory domain that, in the inactive state, sterically hinders the access of substrates to the active site of the kinase. Binding of CaM/S100 to this region causes the autoinhibitory domain to adopt a

new conformation and releases the intrasteric inhibition (Kemp *et al.*, 1994; Goldberg *et al.*, 1996; Kobe *et al.*, 1996). For these kinases, activation can be artificially induced by deletion or proteolysis of the autoinhibitory domain. In contrast, the S100 binding domain of Ndr is N-terminal to the kinase domain, and is not autoinhibitory: deletion of part or all of this domain resulted in a kinase that showed significantly reduced activity (Figure 6 and data not shown). This suggests that S100 proteins activate Ndr by inducing a positive allosteric conformational change rather than by relieving an autoinhibited conformation.

Like CaM, S100 proteins are likely to regulate multiple target proteins. Other potential nuclear effector proteins of S100A1 and S100B include p53 (Baudier *et al.*, 1992; Scotto *et al.*, 1998) and bHLH transcription factors (Baudier *et al.*, 1995; Onions *et al.*, 1997). Several cytoskeletal proteins, such as tubulin (Baudier *et al.*, 1982), caldesmon (Fujii *et al.*, 1990) and MAP2 (Donato *et al.*, 1989), have also been identified as possible targets of S100A1 and S100B. S100A1 may additionally regulate myosin activity by activating twitchin kinase, whose catalytic domain is related to those of the myosin light chain kinases (Heierhorst *et al.*, 1997). Thus, the range of effector proteins so far identified for S100A1 and S100B suggests roles in the regulation of transcription, cell-cycle progression and cell morphology (Schäfer and Heizmann, 1996). Indeed, in studies using antisense oligonucleotides or antisense RNA to inhibit S100B expression in glioma cells, depletion of S100B caused a reduction in proliferative rate to ~30% of that of control cells, and also induced a flattened cell morphology in which cells lacking S100B occupied an ~4-fold larger area than control cells (Selinfreund *et al.*, 1990). Similarly, loss of S100A1 caused a decrease of growth and altered neurite organization in PC12 cells (Zimmer *et al.*, 1998). Two studies have shown that S100B protein levels are subject to cell-cycle regulation (Fan, 1982; Marks *et al.*, 1990). It is, therefore, interesting to note that the catalytic domain of Ndr is closely related to those of several protein kinases (Orb6, Wts, LET-502 and Dbf2) identified in genetic screens as being critical for the regulation of cell division and cell shape.

Expression levels of S100A1 and S100B are often altered in human cancers (Ilg *et al.*, 1996). Most notably, S100B is highly overexpressed in melanoma cells relative to untransformed melanocytes (Cocchia *et al.*, 1981; Marks *et al.*, 1990), permitting the use of S100B as a marker for melanoma (Cochran *et al.*, 1993). Our findings suggest that Ndr may be hyperactivated in a subset of S100B-positive melanomas and that, in such cases, antagonism of S100B can reverse this hyperactivation. Note, however, that several S100B-positive melanomas did not show elevated Ndr activity. This would be consistent with a model in which Ca²⁺/S100B is one of several cellular regulators of Ndr, such that S100B overexpression can cause hyperactivation of Ndr given that an additional mutation also occurs, e.g. loss of function of a negative regulator of Ndr. Nevertheless, the link between S100B and Ndr could be of clinical interest if a causal relationship between S100B overexpression and the development of melanoma were to be established in the future. In this respect it is noteworthy that forced overexpression of

S100A4, which is closely related to S100B, is sufficient to induce a metastatic phenotype in normally non-metastatic mammary epithelial cells (Grigorian *et al.*, 1996; Lloyd *et al.*, 1998).

Materials and methods

Antibodies

A polyclonal antibody (Ab¹⁻⁴⁶⁵) against human Ndr was generated by injecting rabbits with GST-Ndr (Millward *et al.*, 1995). The antiserum was affinity purified using columns of GST followed by GST-Ndr essentially as described previously (Koff *et al.*, 1992), except that Ndr-specific antibodies were eluted with 0.1 M triethylamine pH 11.5, and subsequently neutralized with 0.2 vol 500 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 3.0. Biotinylated Ab¹⁻⁴⁶⁵ was prepared by reaction of the purified antibodies with biotin amidocaproate *N*-hydroxysuccinimide ester (Sigma) as described previously (Harlow and Lane, 1988). For immunoprecipitation prior to immunoblotting, IgG was isolated from the immune serum using protein A-Sepharose, rebound onto fresh protein A-Sepharose, and then covalently cross-linked using dimethyl pimelimidate (Pierce). Antibodies which recognize a C-terminal peptide of human Ndr (Ab⁴⁵²⁻⁴⁶⁵) were used as reported previously (Millward *et al.*, 1995). A polyclonal antiserum which recognizes S100B was raised using recombinant human S100B (see below) as antigen, and was used without further purification. The 12CA5 monoclonal antibody was used for detection and immunoprecipitation of HA-Ndr.

Plasmids

Mammalian expression plasmids encoding untagged Ndr (pECE-Ndr-WT, pECE-Ndr-Δ65-81, pECE-Ndr-Δ1-84) have been described previously (Millward *et al.*, 1995). Plasmids expressing HA epitope-tagged Ndr (pECE-HA-Ndr-WT, pECE-HA-Ndr-Δ65-81) were constructed by amplifying the respective untagged pECE constructs with primers 5'-GGGGTACCACCATGGCATACCCCTACGACGTGCCCGACTATGCGCACAGGCTCAACACCTTGC-3' and 5'-GCTCTAGACTATTTTGTGCTTTTCATGTAG-3'. The PCR products were cloned between the *KpnI* and *XbaI* sites of pECE. The sequence of each construct was confirmed prior to transfection. The S100B cDNA was amplified from reverse-transcribed human cerebellum RNA using primers based on the published sequence (Allore *et al.*, 1990), and cloned into *HindIII/XbaI*-cut pBluescript. The insert was then excised with *KpnI* and *XbaI* and ligated into the corresponding sites of pECE.

Cell culture

Cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. M960616 cells were a generous gift from Dr R. Dummer (Dermatologische Klinik, Universitätsspital Zürich); other cell lines were from the American Type Culture Collection. COS-1 kidney fibroblasts were transfected using DEAE-dextran (Seed and Aruffo, 1987) and were used 72 h after transfection. In some experiments, cells were treated with 20 µM A23187 (Sigma) or with 50 µM W-7 (Calbiochem) prior to collection.

CaM and S100B binding assays

For binding assays using purified recombinant Ndr, 2.5 µg of either GST or GST-Ndr (Millward *et al.*, 1995) were diluted to 0.1 µg/µl in TBST (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.05% Tween 20) and mixed with 20 µl affinity resin equilibrated in the same buffer. Either CaM-agarose (Sigma) or S100B-agarose was used. S100B-agarose was prepared by reaction of bovine S100B (Sigma) with Affi-gel 15 (Bio-Rad); both CaM- and S100B-agarose contained 1.5 mg protein per ml of beads. Binding reactions and subsequent wash buffers were supplemented with 1 mM CaCl₂ or 1 mM EGTA as indicated. After mixing at 4°C for 2 h, the beads were spun down and the supernatant removed and saved. The beads were washed three times with the binding buffer and then eluted with TBST containing 5 mM EGTA. Equal portions of input, unbound and EGTA-eluted fractions were analysed by immunoblotting with Ab⁴⁵²⁻⁴⁶⁵ or with a polyclonal anti-GST antiserum (1:1000).

COS-1 cells expressing wild-type Ndr, Ndr-Δ65-81 or Ndr-Δ1-84 were scraped into ice-cold phosphate-buffered saline (PBS), spun down and mechanically homogenized in buffer A [50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 4 µM leupeptin and 1 mM benzamide] containing 1 mM CaCl₂. Extracts (20 µg, 1 mg/ml) were mixed for 2 h with 6 µl

CaM-agarose. After washing three times with 1 ml buffer A containing 1 mM CaCl₂, the beads were eluted with buffer A containing 5 mM EGTA. Equal portions of the unbound and EGTA-eluted fractions were analysed for Ndr by immunoblotting with Ab⁴⁵²⁻⁴⁶⁵.

GST-Ndr kinase assay

Typically, 1 µg GST-Ndr WT or GST-Ndr K118A (in solution or immobilized on glutathione-agarose beads, as indicated) was assayed in a 20 µl reaction containing 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 mM DTT, 100 µM [γ -³²P]ATP (~0.3 µCi/µl) and 1 mM Ndr substrate peptide (KKRNRRLSVA). In Figure 1, various substrate peptides were used at 0.5 mM, as indicated. In other experiments, assays were supplemented with CaCl₂, EGTA and calcium-binding proteins, as indicated in the figure legends. After incubation for 1 h at 30°C (during which time phosphate incorporation into peptides was linear), supernatants were removed and spotted onto 2 cm² squares of P81 phosphocellulose paper (Whatman), which were then washed 5 × 5 min in 1% phosphoric acid and once in acetone, before counting in a liquid scintillation counter. One unit of Ndr activity was defined as that amount which catalyses the phosphorylation of 1 nmol substrate in 1 min. Recombinant human S100A1, S100A2, S100A4, S100A6 and S100B have been described previously (Pedrocchi *et al.*, 1994; Ilg *et al.*, 1996). Bovine CaM and bovine S100B homodimer were from Sigma.

HA-Ndr kinase assay

Transfected COS-1 cells were washed with ice-cold PBS and lysed on the plate with 1 ml IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 0.5 mM EGTA, 20 mM β-glycerophosphate, 50 mM NaF, 0.5 mM PMSF, 4 µM leupeptin, 1 mM benzamide and 1 mM Na₃VO₄) supplemented with 1 µM microcystin. Lysates were centrifuged at 14 000 g for 20 min. Duplicate aliquots of supernatant (250 µg, 0.5 mg/ml) were mixed for 3 h at 4°C with 12CA5 prebound to protein A-Sepharose (~1 µg antibody bound to 2 µl beads; Pharmacia). The beads were then washed twice with IP buffer, once with IP buffer containing 1 M NaCl, once again with IP buffer, and finally twice with 20 mM Tris-HCl pH 7.5 containing 4 µM leupeptin and 1 mM benzamide. Beads were resuspended in 30 µl buffer containing 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 100 µM [γ -³²P]ATP (~0.1 µCi/µl), 1 mM Ndr substrate peptide (KKRNRRLSVA), 1 µM cAMP-dependent protein kinase inhibitor peptide (PKI; purchased from Bachem, Bubendorf, Switzerland), 4 µM leupeptin, 1 mM benzamide and 1 µM microcystin. After 60 min at 30°C, 20 µl of supernatant were removed, and phosphate incorporation into the peptide quantitated as for GST-Ndr.

Immunodetection of endogenous Ndr

Cell monolayers were washed with ice-cold PBS and then scraped from the plate into ice-cold PBS, spun down and lysed in ~4 cell volumes of IP buffer containing 1 µM microcystin. After 30 min on ice, the lysate was centrifuged at 14 000 g for 20 min. Supernatants (400 µg, 4 mg/ml) were mixed for 3 h at 4°C with 2 µl protein A-Sepharose containing covalently cross-linked Ab¹⁻⁴⁶⁵. The beads were washed as for HA-Ndr and then eluted by boiling for 3 min in 62.5 mM Tris-HCl pH 6.8 containing 2% SDS and 10% glycerol. The eluate was removed, made to 10 mM DTT and 0.1% bromophenol blue, boiled again and then loaded onto an SDS-polyacrylamide gel for immunoblotting with biotinylated Ab¹⁻⁴⁶⁵.

Assay of endogenous Ndr kinase activity

Cells were harvested and lysed as for immunodetection of endogenous Ndr. The lysates were precleared by incubation for 30 min with 5 µl protein A-Sepharose. Duplicate aliquots of precleared supernatant (400 µg, 4 mg/ml) were then incubated for 2 h on ice with 0.5 µg affinity purified Ab¹⁻⁴⁶⁵. Immune complexes were captured by mixing for 1 h with 2 µl protein A-Sepharose. The beads were washed and assayed as for HA-Ndr. Preincubation of the immunoprecipitating antibody with GST-Ndr bound to glutathione-agarose beads reduced the activity measured in the kinase assay by >90%, whereas preincubation with GST had no effect (data not shown).

Immunodetection of S100B

Cell extracts (1 mg, 4 mg/ml, prepared as for immunodetection of endogenous Ndr) were mixed for 3 h at 4°C with 0.5 µl anti-S100B antiserum prebound to 2 µl protein A-Sepharose. The beads were washed twice with IP buffer and twice with 20 mM Tris-HCl pH 7.5 containing 4 µM leupeptin and 1 mM benzamide, and were then boiled in sample

buffer. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-S100B.

Western blotting

To detect Ndr or HA-Ndr, samples were resolved by 7.5 or 10% SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked in TBSTT (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% Tween 20) containing 5% skimmed milk powder, and were then probed for 2 h with Ab⁴⁵²⁻⁴⁶⁵ (4 µg/ml), Ab¹⁻⁴⁶⁵ (1 µg/ml) or 12CA5 monoclonal antibody (1:100). Bound antibodies were detected with peroxidase-conjugated secondary antibodies and ECL (Amersham). To detect immunoprecipitated Ndr, biotinylated Ab¹⁻⁴⁶⁵ (1 µg/ml in TBSTT containing 5% BSA) was used, followed by streptavidin-peroxidase (Amersham) diluted 1:1000 in the same buffer. To detect immunoprecipitated S100B, samples were separated by 20% SDS-PAGE (Okajima *et al.*, 1993) and transferred to PVDF. The membrane was blocked for 2 h with TBSTT containing 5% BSA and 1% FCS, and was then probed with anti-S100B antiserum diluted 1:1000 in the same buffer.

Co-immunoprecipitation of Ndr and S100B

Transfected COS-1 cells were lysed by preparing hypotonic and high-salt extracts as described (Dignam *et al.*, 1983) except that all buffers were supplemented with 0.5 mM CaCl₂, 0.5 mM PMSF, 4 µM leupeptin, 1 mM benzamidine, 1 µM microcystin and 1 mM Na₃VO₄. The hypotonic and high-salt extracts were combined and centrifuged. Two-hundred micrograms of cell extract (1 mg/ml) were mixed for 3 h at 4°C with 0.5 µl anti-S100B antiserum immobilized on 2 µl protein A-Sepharose. Following this, the beads were washed three times in 20 mM HEPES pH 7.9, 25 mM KCl, 5% glycerol, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM PMSF, 4 µM leupeptin, 1 mM benzamidine, 1 µM microcystin and 1 mM Na₃VO₄. Immunoprecipitates were either boiled in sample buffer and immunoblotted for S100B as described above, or were eluted with IP buffer containing 1 M NaCl and 5 mM EGTA, and the eluates immunoblotted with biotinylated Ab¹⁻⁴⁶⁵.

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