Mechanism of the PII-Activated Phosphatase Activity of *Escherichia coli* NRII (NtrB): How the Different Domains of NRII Collaborate to Act as a Phosphatase†

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ABSTRACT: The phosphatase activity of the homodimeric NRII protein of *Escherichia coli* is activated by the PII protein and requires all three domains of NRII. Mutations in the N-terminal domain (L16R), central domain (A129T), C-terminal domain PII-binding site (S227R), and C-terminal domain ATP-lid (Y302N) of NRII result in diminished phosphatase activity. Here, we used heterodimers formed in vitro from purified homodimeric proteins to study the phosphatase activity. A129T, S227R, and Y302N mutant subunits and A129T/S227R, A129T/Y302N, and S227R/Y302N double-mutant subunits formed stable heterodimers and were amenable to analysis; heterodimers containing these mutant subunits in various combinations were formed and their activities assessed. Complementation of the PII-activated phosphatase activity was observed in heterodimers containing S227R and Y302N subunits and in heterodimers containing A129T and Y302N subunits, but not in heterodimers containing A129T and S227R subunits. Complementation of the PII-activated phosphatase activity was also observed in heterodimers containing A129T/S227R and Y302N subunits, but not in heterodimers containing A129T/Y302N and S227R subunits. Finally, inclusion of an S227R/Y302N subunit in a heterodimer with a subunit having wild-type phosphatase activity resulted in a dramatic decrease in phosphatase activity, while inclusion of an A129T/S227R subunit did not. These results suggest that the phosphatase activity of NRII requires the collaboration of the PII-binding site from one subunit of the dimer, the central domain from the same subunit, and the ATP-lid from the opposing subunit, in addition to the undefined N-terminal domain requirement(s).

Two-component signal transduction systems (TCS) are common in prokaryotes and are also found in lower eukaryotes and plants (reviewed in ref *1*−*3*). These regulatory systems couple the sensation of environmental signals to specific responses using conserved phosphoryl transfer mechanisms. The TCS contain two protein components termed the "transmitter" or sensor histidine kinase and the "receiver" or response regulator. Environmental signals control the phosphorylation and dephosphorylation of the receiver by the transmitter, with the phosphorylation state of the receiver determining its activity. In most cases, the receiver activates or represses the transcription of specific genes when it is phosphorylated. Genomic studies have indicated that many bacteria contain numerous TCS that regulate various aspects of metabolism, development, and pathogenesis. There are approximately 30 such systems in *Escherichia coli* that regulate a variety of metabolic adaptations and various aspects of cell physiology (*4*).

The mechanisms by which the dimeric transmitter proteins bring about the phosphorylation of the receiver (kinase activity) have been studied in some detail in a number of systems. In a typical TCS, a phosphoryl group is transferred from ATP bound to the C-terminal domain of one subunit of the transmitter to a conserved histidine residue in the central domain of the opposing subunit by a *trans*-intramolecular mechanism (autophosphorylation activity), and then transferred from the phosphorylated histidine to a conserved aspartate residue in the receiver (*5*−*8*). This flow of phosphoryl groups from ATP to the receiver may be regulated in response to environmental signals sensed by the transmitter (*9*). In other cases, more elaborate phosphorelay systems are employed that involve transfer of the phosphoryl group from transmitter to receiver and then from the receiver to a phosphotransferase protein which in turn transfers the phosphoryl group to a final receiver protein (*10*).

The phosphorylation state of the receiver may also be regulated by its dephosphorylation, but the molecular mechanisms by which transmitter proteins bring about receiver dephosphorylation are less well-defined. The receiver proteins often possess "autophosphatase" activities, with half-lives of the acyl phosphate ranging from seconds to hours, depending on the receiver (*11*). In some cases, the transmitter protein, in response to environmental signals, brings about the very rapid dephosphorylation of the phosphorylated

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[1] Abbreviations: PII, signal transduction protein encoded by *glnB*; NRII, NtrB, signal transduction protein encoded by *glnL* (*ntrB*); NRI, NtrC, signal transduction protein encoded by *glnG* (*ntrC*); NRI-N, N-terminal domain of NRI consisting of amino acids 1−118 of NRI; MBP, maltose binding protein of *E. coli*; MBP-NRII, NRII fused to the C-terminus of MBP; MBP-CT111, amino acids 111−349 of NRII fused to the C-terminus of MBP; MBP-CT126, amino acids 126−349 of NRII fused to the C-terminus of MBP; BSA, bovine serum albumin; AMP-PNP, adenosine 5′-(β, γ-imido)triphosphate; TFPAM-3, N-(4-azido-2,3,5,6-tetraflourobenzyl)-3-maleimidopropionamide.

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receiver (*12*). This activity has been referred to as the regulated phosphatase activity (*13*), although it is not known whether it represents a unique activity or the activation of the receiver autophosphatase activity. Here, we refer to this activity of the transmitter as the phosphatase activity. Genetic and physiological analysis of several TCS indicates that this activity plays a central role in the regulation of receiver activity (reviewed in ref *14*). In some TCS, phosphatase activities are found in additional proteins, unrelated to the transmitter protein (reviewed in ref *15*).

The transmitter proteins of TCS are typically composed of an unconserved N-terminal domain linked to the conserved transmitter module that forms the central and C-terminal domains (reviewed in ref *16*). The lack of conservation among the N-terminal domains of the transmitter may reflect the need for the various TCS to respond to distinct signals, with the N-terminal domain providing a sensory function. Many transmitter proteins are membrane-bound proteins, and in some cases, transmembrane signaling has been implicated in the control of the activities of the cytoplasmic transmitter module (*17*, *18*). In other cases, sensation involves cytoplasmic domains located immediately adjacent to the conserved transmitter module (*19*, *20*), is due to sensory properties of the transmitter module itself (*21*), or is due to the interaction of additional signal transduction proteins with the cytoplasmic transmitter module (*22*, *23*). These regulatory interactions may control the transmitter kinase or phosphatase activity or both, depending on the system, to control the phosphorylation state of the receiver.

The NRII/NRI (NtrB/NtrC) TCS of *E. coli* controls the expression of genes belonging to the Nitrogen (Ntr) regulon in response to intracellular signals of nitrogen and carbon availability (reviewed in ref *24*). The transmitter NRII phosphorylates and dephosphorylates the receiver NRI. NRII is a cytoplasmic transmitter protein that consists of a small N-terminal domain containing a PAS motif linked to its central and C-terminal domain that together comprise the conserved transmitter module. PAS motifs have been shown to bind regulatory ligands (*25*) or in some cases are involved in mediating protein−protein interactions (*26*). NRII autophosphorylation occurs at histidine 139 in the central domain. The phosphoryl group is then transferred to aspartate 54 in the N-terminal receiver domain of NRI. The phosphorylated form of NRI (NRI∼P) activates the initiation of transcription of σ^{54}-dependent Ntr genes (*12*, *27*).

Genetic and physiological studies have indicated that the phosphatase activity of NRII is the primary target of regulatory signals (*28*, *29*). This regulation is carried out by the PII protein. The homotrimeric PII protein is a member of a large family of signal transduction proteins that are found throughout nature, with representatives in prokaryotes, lower eukaryotes, and plants (reviewed in ref *30*). The binding of PII to NRII results in a modest inhibition of the autophosphorylation of NRII and a dramatic activation of the NRII phosphatase activity (*12*, *31*). The ability of PII to regulate NRII is controlled by the covalent modification of PII in response to the nitrogen signal glutamine (*32*) and by the binding of the allosteric effector, 2-ketoglutarate (the carbon signal) to PII (*33*−*35*). In the presence of saturating ATP, the binding of a single molecule of 2-ketoglutarate to the PII trimer exhibits negative cooperativity such that binding of additional 2-ketoglutarate molecules is disfavored. The optimal form of PII for bringing about the regulation of NRII is the unmodified, asymmetric trimer with three ATP molecules and one 2-ketoglutarate molecule bound.

A large exposed loop of PII known as the T-loop is responsible for the interaction of PII with NRII (*36*). Heterotrimeric PII molecules that contained a single wild-type subunit and two subunits that had a deletion of the T-loop retained the ability to elicit the phosphatase activity of NRII (*37*), suggesting that a single T-loop of the PII trimer was sufficient for binding NRII. In addition, cross-linking agents uniquely positioned at sites in the PII T-loop are specifically cross-linked to NRII in an ATP and 2-ketoglutarate dependent manner (*23*). The purified cross-linked PII−NRII complex, which exhibited phosphatase activity, consisted of the PII trimer covalently linked to a single subunit of the NRII dimer (*23*).

Genetic and biochemical analysis of the phosphatase activity of NRII indicated that this activity is not a reversal of the phosphotransfer reaction and that the role of PII is strictly regulatory (*38*). Studies with purified truncated derivatives of NRII indicated that the phosphatase activity resides in the central domain of NRII and suggested that the phosphatase activity requires a particular conformation of NRII (*38*). PII binds to the C-terminal domain of NRII (*23*) and apparently causes the central domain to adopt the conformation with potent phosphatase activity; this intramolecular signal transduction requires the presence of the N-terminal domain of NRII (*38*).

Numerous mutations affecting the PII-activated phosphatase activity of NRII have been identified, and these mutations map in all three domains of NRII. Specifically, mutations mapping within the N-terminal domain of NRII, throughout the central domain, on two distinct surfaces of the C-terminal domain, and in the linkers connecting the domains, result in reduction of the NRII phosphatase activity while having little or no effect on kinase activity (*39*, *40*). Of these, a mutation within the N-terminal domain (L16R), at the beginning of the central domain (A129T), and at each of the two surfaces of the C-terminal domain (S227R and Y302N) were selected for in vitro studies of the purified proteins (*40*). The Y302N mutation mapped to the "ATP-lid" of the C-terminal domain, and the S227R mutation mapped to the "back" of the C-terminal domain. The S227R protein was a defective phosphatase due to its inability to bind PII. The L16R, A129T, and Y302N proteins were defective phosphatases but retained normal or nearly normal PII-binding. The A129T, S227R, and Y302N mutant proteins displayed normal autophosphorylation kinetics, suggesting normal interactions with ATP and ADP (*40*). The biochemical data available for NRII, in conjunction with structural information available for other TCS transmitter proteins (*41*−*44*), led us to propose a model for the domain arrangement in the NRII dimer (*40*, *45*) as shown in Figure 1.

Since mutations spread throughout the NRII protein can affect the phosphatase activity, we were interested to determine how the domains of NRII collaborate to function as a phosphatase in the presence of PII. Here, we describe the use of purified single or double-mutant NRII proteins and incorporation of their subunits into heterodimeric proteins to study the mechanism of the PII-activated phosphatase activity of NRII. We show that in certain cases, the inclusion

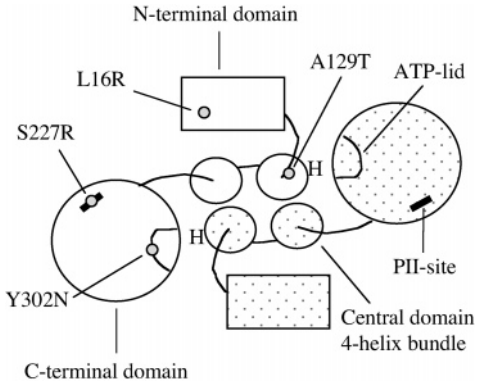

FIGURE 1: Model for the domain arrangement of the NRII dimer showing the positions of mutations used in this study. The model is similar to those in references (*40*, *45*) and is based on structural information available for related two-component system transmitter proteins (*41*−*44*) and biochemical studies of NRII. Each NRII subunit is composed of three domains. The N-terminal domain is unique to NRII proteins and is important for intramolecular signal transduction. The central domain is the dimerization domain containing the site of autophosphorylation and harbors the phosphotransfer and phosphatase activities. It is presumed to consist of two adjacent α-helices separated by a hairpin loop that, together with the central domain of the opposing subunit, make up a putative four-helix bundle. The active site histidine (His139), which resides in the first putative helix, is shown labeled as an "H". The C-terminal domain is responsible for PII- and ATP-binding and is presumed to exhibit the conserved α/*β* sandwich fold common to the ATP-binding domain of other transmitter proteins. This domain contains the ATP-binding site, a loop over the ATP-site termed the ATP-lid, and the PII-binding site on the "back" side. In the picture, the dimer is shown viewed down the four-helix bundle. The N- and C-terminal domains connect to the central domain above the plane of the page, while the two α-helices of a central domain connect below the plane of the page. The proposed positions of amino acid substitutions relevant to this study are shown as dots with their identity labeled. The L16R, A129T, and Y302N mutations result in diminished phosphatase activity while retaining PII-binding ability, whereas the S227R mutation results in decreased phosphatase activity due to severely diminished PII-binding.

of two different mutant subunits within a heterodimer can result in intramolecular complementation that partially restores the PII-activated phosphatase activity. In another case, we show that a double-mutant subunit, when combined with a subunit with wild-type phosphatase activity in a heterodimer, prevents the PII-activated phosphatase activity. The results suggest a model for how the domains of NRII collaborate in the phosphatase activity.

MATERIALS AND METHODS

Plasmid Construction. DNA manipulations were by standard procedures (*46*). Construction of plasmids overexpressing the NRII double-mutant proteins A129T/S227R and A129T/Y302N involved a cloning strategy making use of two conveniently located *SalI* restriction sites in the overexpression plasmids for the single mutant proteins, one between codon 129 and codons 227 and 302 and the other outside of the NRII coding sequence in the expression plasmid. Plasmid pLOP15 (*6*) [encoding the A129T/I221V protein] was digested to remove the *SalI-SalI* fragment covering the distal $\sim^1/_3$ of the NRII coding sequence and containing the I221V mutation. The A129T-containing fragment was used as the "vector" for ligation with the *SalI-SalI* fragment from pAP073 (*40*) [containing the S227R mutation] or from pAP076 (*40*) [containing the Y302N mutation]. The resulting plasmids, pAP125 (encoding A129T/S227R) and pAP127 (encoding A129T/Y302N), were sequenced to ensure that only the correct combination of mutations was present. Sequencing of plasmid DNA was performed using sequencing primers described previously (*39*) by the University of Michigan DNA Sequencing Core Facility. Plasmid pAP123 (encoding S227R/Y302N) construction made use of two convenient *SspI* sites, one located between codons 227 and 302 and the other in the expression plasmid outside of the NRII coding sequence. Plasmid pAP076 (*40*) was digested with *SspI*, and the Y302N-containing fragment was used as the vector for blunt-end ligation with the S227R-containing *SspI*−*SspI* fragment from pAP073 (*40*). The resulting plasmid was sequenced to ensure that it contained the proper combination of mutations.

Purified Proteins. The preparations of PII, PII (E44C/C73S) labeled with the cross-linking reagent TFPAM-3 (Molecular Probes), and NRI-N (consisting of amino acids 1−118 of NRI) were described previously (*23*, *40*, *47*). The fusion proteins MBP-NRII (NRII linked to the C-terminus of maltose binding protein), MBP-CT111 (amino acids 111−349 of NRII linked to the C-terminus of MBP), MBP-CT126 (amino acids 126−349 of NRII linked to the C-terminus of MBP), and the CT111 and CT126 proteins (generated by proteolytic cleavage of the corresponding MBP-fusion proteins and removal of the MBP) were described previously (*6*, *38*, *48*). The preparations of wild-type NRII, NRII (I141V), NRII (A129T), NRII (S227R), and NRII (Y302N) were described previously; each of these proteins was purified in its native form (*40*). The preparation of NRII (L16R) used in this study was previously described (40). This protein was purified as a histidine-tagged fusion protein, and the histidine tag was removed by proteolytic digestion; the protease cleaved product retains three additional amino acids (Gly-Ser-His) at the N-terminus of the protein (*40*). The NRII double-mutant proteins, A129T/S227R, S227R/Y302N, and A129T/Y302N, were purified by the method described previously with the S227R-containing proteins receiving the same considerations as the S227R protein (*40*). This involved overexpression of the proteins in *E. coli* strain RB9132 (Δ *glnL*) [*28*], followed by purification at 4 °C involving ammonium sulfate precipitation, and chromatography on ethyl agarose (Sigma) and Bio-gel A-0.5M (Bio-Rad). In addition, the A129T/Y302N protein was chromatographed on a DE52 (Whatman) ion-exchange column as described (*40*). Protein concentrations were determined by the method of Bradford (*49*) and are stated in terms of the monomer for NRI-N, the dimer for NRII and NRII derivatives, and the trimer for PII.

Preparation of ^{32}P-Labeled NRI-N∼P. [^{32}P]NRI-N∼P was prepared similar to a previously described method used for full-length NRI (*38*). The MBP-CT111 protein was used as the kinase for phosphorylation of NRI-N. MBP-CT111 completely lacks phosphatase activity while retaining kinase activity and does not undergo spontaneous subunit exchange or form stable heterodimers (*38*, *48*, this study). A reaction containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM [γ-^{32}P] ATP, 48 μM NRI-N, and 0.6 μM MBP-CT111 was incubated at 25 °C for 35 min. The [^{32}P]-NRI-N∼P was separated from nucleotides on a PD10 Sephadex G-25 column (Pharmacia) equilibrated in 50 mM

Tris-HCl, pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 2 mM EDTA, 1 mM DTT, and stored at −20 °C.

Polyacrylamide Gel Electrophoresis. Nondenaturing and SDS-polyacrylamide gel electrophoresis were carried out as described previously (*6*, *50*). The nondenaturing gels were run at 150 V at 4 °C for 3−3.5 h. Gels were stained with Coomassie Brilliant Blue R-250.

Heterodimer Formation and Analysis of Activities. Formation of NRII heterodimers by spontaneous subunit exchange was as previously described (*38*). The indicated combinations of NRII proteins (6 μM total dimer, ratios as indicated) were incubated for 3 h at 37 °C in reactions containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$. Formation of heterodimers by the urea-dialysis procedure was similar to a previously described method (*6*). These reactions were the same as those for spontaneous subunit exchange, except they also contained 2.8 M urea and were incubated for 3 min at 30 °C, followed by dialysis against 4000 volumes of 50 mM Tris-HCl, pH 7.5, 100 mM KCl for 1.5 h at 4 °C. After dialysis the protein concentrations were determined by the method of Bradford (*49*). Heterodimer formation was monitored by analysis on nondenaturing 10% polyacrylamide gels.

Enzymatic activities of the NRII heterodimers were monitored using the assays described below, with the "heterodimer" and "control" samples prepared as follows. In most experiments the heterodimer sample was prepared by mixing two different homodimers (as indicated) in a 1:1 molar ratio (6 μM total dimers) and subjecting the mixture to the urea-dialysis procedure. The theoretical equilibrium distribution of NRII molecules in this sample is 25% each homodimer and 50% heterodimer, assuming random reassociation of the NRII subunits. The activity of this sample was compared to a control sample containing the same total amount of NRII dimers, but which was prepared by subjecting the two different homodimers individually (6 μM total homodimer) to the urea-dialysis procedure followed by mixing of the two homodimers in a 1:1 molar ratio resulting in a sample containing 50% each homodimer (assuming no spontaneous subunit exchange effects). In a few experiments the heterodimer samples were prepared by mixing two different homodimers (as indicated) in a 9:1 molar ratio (6 μM total dimers) and subjecting the mixture to the urea-dialysis procedure. The theoretical equilibrium distribution of NRII molecules in this sample is 81% major homodimer, 1% minor homodimer, and 18% heterodimer, assuming random reassociation of the NRII subunits. In this case the control samples were prepared as for the 1:1 ratio, except that after the urea-dialysis procedure the two different homodimers were mixed in a 9:1 molar ratio, resulting in a sample containing 90% major homodimer and 10% minor homodimer (assuming no spontaneous subunit exchange effects).

Since the spontaneous exchange of NRII subunits occurs slowly at low temperatures (*38*), the urea-dialysis treated samples (heterodimer sample and two individually treated homodimer samples) were stored on ice after dialysis. The control samples were prepared by mixing the two urea-dialysis-treated homodimers together on ice just before beginning the activity assays. This minimized the amount of heterodimer that could be formed in the control samples by spontaneous subunit exchange. Immediately after the control sample was prepared, the heterodimer and control samples were prewarmed to the assay reaction temperature (as indicated) for 3 min before starting the assays.

NRII Autophosphorylation Assays. These assays were as previously described (*40*). Errors in the quantitative assay were <10%. The qualitative assay allows us to determine the fraction of our protein preparation that is active, since the phosphorylated NRII dimer exhibits faster mobility than unphosphorylated NRII on nondenaturing polyacrylamide gels (*51*).

Combined Kinase and Phosphatase Assay. This assay was similar to those previously described (*12*, *38*, *40*). Here, PII was added to the reactions from the start; thus, in this assay the final steady-state level of NRI-N∼P reflects the balance of the kinase and phosphatase activities of NRII (*38*). Errors using this assay were <10%, and day-to-day reproducibility was very good. An example of the reproducibility is provided by comparing the wild-type NRII ± PII controls among the panels in Figure 6.

Direct Phosphatase Assay. This assay was similar to one previously described (*38*), except that the N-terminal domain of NRI (NRI-N) was used in place of NRI, and the substrate [^{32}P]NRI-N∼P was present at 1.13 μM. The data shown here were obtained using two separately prepared batches of [^{32}P]-NRI-N∼P and were highly consistent. Errors using this assay were <5%, and day-to-day reproducibility was good, as evidenced by comparing the PII ± wild-type NRII data among the figures. For plotting the data the starting level of [^{32}P]NRI-N∼P (zero time point) was determined by a best fit of the PII alone data to a simple exponential decay function.

Cross-Linking Reactions. The binding of PII to NRII was assessed using a cross-linking assay performed as described previously (*23*, *40*).

RESULTS

Subunit Exchange Activity of Phosphatase-Deficient NRII Proteins. We sought to determine if PII-activated phosphatase activity can be restored to phosphatase-deficient mutant NRII proteins by including different mutant subunits in NRII heterodimers. Toward this end, we examined the ability of different phosphatase deficient NRII proteins to form stable heterodimers. Previous results showed that the simple mixing of a homodimeric mutant NRII protein that contains a substitution of valine for isoleucine at residue 141, NRII (I141V), with a homodimeric NRII protein consisting of NRII linked to the C-terminus of maltose binding protein (MBP-NRII) resulted in the formation of the heterodimer species, with the equilibrium position reached in approximately 3 h at 37 °C (*38*). The I141V protein exhibits slightly elevated kinase activity, while retaining wild-type phosphatase activity in the presence of PII (*40*); this protein will be used in some of the experiments described here. We examined the ability of wild-type NRII and the L16R, A129T, S227R, and Y302N mutant proteins to form stable heterodimers with MBP-NRII by spontaneous subunit exchange (Materials and Methods). The larger size of the MBP-NRII fusion protein allows the homodimer and heterodimer species to be resolved by nondenaturing polyacrylamide gel electrophoresis (*6*). The wild-type, A129T, S227R, and Y302N proteins were all capable of forming stable het-

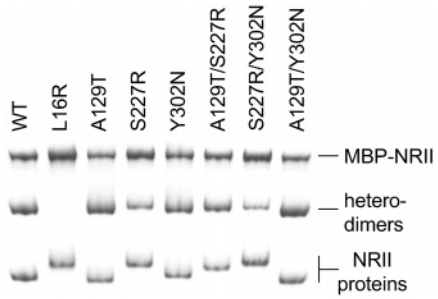

FIGURE 2: Spontaneous subunit exchange activity of NRII dimers. Wild-type NRII or mutant forms of NRII were mixed with MBP-NRII (3 μM each homodimer) in reactions containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$. After incubation for 3 h at 37 °C aliquots of the reaction mixtures were analyzed on a nondenaturing 10% polyacrylamide gel stained with Coomassie Brilliant Blue R-250. Positions of the homodimers and heterodimers are indicated to the right of the gel, and the identities of the NRII proteins are listed above the gel lanes.

erodimers with MBP-NRII, while the L16R protein did not detectably form heterodimers (Figure 2). Figure 2 also shows the results obtained with the NRII double-mutant proteins that will be described in detail later; as shown, these proteins also were able to form heterodimers with MBP-NRII.

The L16R protein was further examined in spontaneous subunit exchange experiments using a 1:1 molar ratio of the L16R protein versus the I141V, A129T, and Y302N proteins. These proteins can be separated on nondenaturing polyacrylamide gels since the L16R protein exhibits slower mobility due to the positive charge introduced by the arginine side chain. Heterodimer formation was not detected (data not shown). We also tried using a 10-fold molar excess of the MBP-NRII protein over the L16R protein (and vice versa) and did not detect any heterodimer formation (data not shown).

Previous experiments have shown that NRII heterodimers can also be formed by treating a mixture of two homodimers with urea to dissociate the subunits followed by dialysis to remove the urea and allow reassociation and re-assortment of subunits (*6*). In those experiments 2.8 M urea was the minimal concentration that permitted dissociation of the NRII subunits while not affecting the ability of the subunits to bind ATP. We attempted to form heterodimers containing the L16R protein using the urea-dialysis procedure (Materials and Methods). We tested 1:1 molar ratios of the L16R protein versus the I141V, A129T, and Y302N proteins with urea concentrations of 1.4, 2.8, 5, and 6.7 M. In all cases heterodimer formation was not detected with the L16R protein (data not shown).

The results with the L16R protein suggested that it could only form stable homodimers. Our current results are consistent with previous results that indicated that truncated polypeptides of NRII lacking the N-terminal domain, designated CT111 and CT126, were unable to form heterodimers by spontaneous subunit exchange, while a truncated polypeptide consisting of just the N-terminal domain and the central domain was capable of spontaneous subunit exchange (*38*). Here, the ability of the truncated versions of NRII lacking the N-terminal domain to form stable heterodimers using the urea-dialysis procedure with 2.8 M urea was examined. We tested 1:1 molar ratios of the MBP-CT111 and MBP-CT126 proteins (in which the transmitter module of NRII is fused to the C-terminus of MBP) versus the I141V protein and did not detect any heterodimer formation (data not shown). In addition, we tested 1:1 molar ratios of the CT111 and CT126 proteins (MBP removed by protease digestion, [*38*]) versus the L16R, A129T, S227R, and Y302N proteins and did not detect any heterodimer formation (data not shown).

Intersubunit Complementation of Phosphatase Activity in NRII Heterodimers. The results presented thus far demonstrated that stable NRII heterodimers containing subunits from the wild-type, A129T, S227R, and Y302N proteins could be formed. We examined whether subunits containing the A129T, S227R, and Y302N mutations, when placed in heterodimeric NRII molecules, could complement and restore PII-activated phosphatase activity to the NRII heterodimers. Heterodimers were formed by the urea-dialysis procedure (2.8 M urea), since this method ensures that the equilibrium position will be obtained by a standard treatment for all of the proteins.

The NRII heterodimer species cannot be isolated and studied in the absence of the homodimer species since any purified preparation of heterodimer would form homodimers by spontaneous subunit exchange. Thus, the heterodimer must be studied in the presence of the homodimers. As a control for this, we compare the activities of two mixtures of NRII dimers that have the same total amount of NRII dimers, but their composition varies in terms of the fraction that is homodimer versus heterodimer. The heterodimer sample consists of a mixture of a single heterodimer species and two homodimer species, and the corresponding control sample contains predominantly the two homodimer species with very little of the heterodimer species formed. The preparation of these heterodimer and control samples and their predicted compositions is described in Materials and Methods.

The activities of heterodimer and control samples for all three possible combinations of mutations (S227R with Y302N, A129T with S227R, and A129T with Y302N) were examined in a combined kinase and phosphatase assay described previously (*38*, *40*). In this assay the kinase and phosphatase activities of NRII are assessed in the absence or presence of PII with the N-terminal receiver domain of NRI (NRI-N) serving as the substrate for phosphorylation and dephosphorylation (Materials and Methods). PII is added to the reactions from time zero (where indicated), and the final steady-state level of phospho-NRI-N (NRI-N∼P) reflects the balance of the kinase and phosphatase activities of NRII (*38*).

Figure 3A shows the results obtained with heterodimer and control samples of the S227R and Y302N proteins. A nondenaturing polyacrylamide gel analysis demonstrated that our heterodimer sample contained the S227R::Y302N heterodimer species in roughly the predicted amount while the control sample contained little or no S227R::Y302N heterodimers (Figure 3A inset). [The double colon is used to refer to the heterodimer species; double-mutant proteins that will be described later are designated with a forward slash between the two mutations.] The activities of equal amounts (0.3 μM total NRII dimer) of the two samples were assessed in the absence or presence of 0.3 μM PII trimer. In the presence of PII, the control sample resulted in a lower steady-state level of NRI-N∼P than that in the absence of PII reflective of the residual phosphatase activities of the S227R

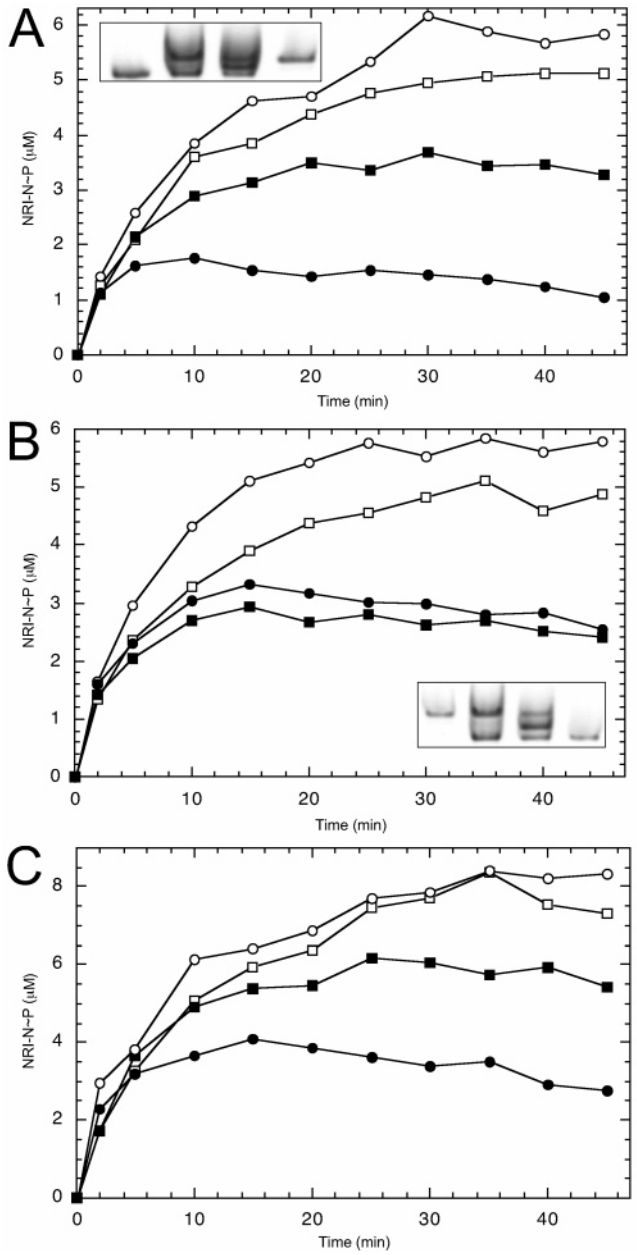

FIGURE 3: Combined kinase and phosphatase assay of NRII heterodimers containing single amino acid substitutions in trans. (A) Heterodimer and control samples were prepared by the urea-dialysis procedure using a 1:1 molar ratio of the S227R and Y302N proteins (Materials and Methods). The activity of the samples was measured at 25 °C in reactions containing 0.3 μM total NRII dimer (control or heterodimer sample) in the absence or presence of PII (0.3 μM) and also containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM [γ-^{32}P]ATP, 50 μM 2-ketoglutarate, and 30 μM NRI-N. Symbols: control sample − PII (□), control sample + PII (■), heterodimer sample − PII (○), heterodimer sample + PII (●). The inset shows a nondenaturing 10% polyacrylamide gel analysis of the samples used in the assay. Lanes (left to right): 1 μg Y302N, 3.5 μg control sample, 3.5 μg heterodimer sample, 1 μg S227R. (B) Heterodimer and control samples were prepared using a 1:1 molar ratio of the A129T and S227R proteins and assayed as in panel A except that PII (when present) was 0.2 μM. Symbols are the same as those in panel A. The inset shows a nondenaturing 10% polyacrylamide gel analysis of the samples used in the assay. Lanes are (left to right) as follows: 1 μg S227R, 3.5 μg control sample, 3.5 μg heterodimer sample, 1 μg A129T. (C) Heterodimer and control samples were prepared using a 1:1 molar ratio of the A129T and Y302N proteins and assayed as in panel A except that PII (when present) was 0.2 μM. Symbols are the same as those in panel A.

and Y302N homodimers (Figure 3A). In contrast, in the presence of PII the heterodimer sample resulted in a significantly lower steady-state level of NRI-N~P than the control sample in the presence of PII, while the kinase activity of the heterodimer sample in the absence of PII was slightly elevated. This indicates that the PII-activated phosphatase activity may have been restored to some extent in the S227R::Y302N heterodimers.

Figure 3B shows the results using the A129T and S227R proteins. The activity of equal amounts (0.3 μM total NRII dimer) of the samples was assessed as above, except that the PII concentration was 0.2 μM trimer. The A129T protein is not as severely defective for the phosphatase activity as the S227R and Y302N proteins and retains significant phosphatase activity under our assay conditions at 0.3 μM PII (*40*). The defect of the A129T protein is more pronounced when assayed at lower PII concentrations (0.2 μM), while the wild-type NRII protein still exhibits potent phosphatase activity under these conditions (*40*). The heterodimer sample exhibited slightly higher kinase activity in the absence of PII than the control sample and resulted in a steady-state level of NRI-N~P in the presence of PII similar to the control sample (Figure 3B). This suggests that the PII-activated phosphatase activity was not restored in the A129T::S227R heterodimers.

Figure 3C shows the results with the A129T and Y302N proteins. In this case we were unable to resolve the heterodimer species from the homodimer species by nondenaturing polyacrylamide gel electrophoresis (data not shown). Nonetheless, the results show that the heterodimer sample resulted in a significantly lower steady-state level of NRI-N~P in the presence of PII (0.2 μM) than the control sample, while the kinase activity of the two samples in the absence of PII was essentially the same (Figure 3C). This indicates that the phosphatase activity may have been restored to some extent in the A129T::Y302N heterodimers.

The results presented thus far have suggested that the S227R::Y302N and A129T::Y302N heterodimers had either restored phosphatase activity in the presence of PII, restored inhibition of kinase activity in the presence of PII, or some combination of the two, while having normal or slightly elevated kinase activity in the absence of PII. Similar results with all three combinations of mutations were obtained in experiments where heterodimers were formed by the spontaneous exchange of subunits in the absence of urea (data not shown). To distinguish between phosphatase activity and inhibition of kinase activity, we directly assayed phosphatase activity of the heterodimer and control samples in the absence of competing kinase activity in reactions containing ^{32}P-labeled NRI-N~P as the substrate and the non-hydrolyzable ATP analogue AMP-PNP in place of ATP (Materials and Methods).

Figure 4 shows the results for all three combinations, 1:1 S227R with Y302N (Figure 4A), 1:1 A129T with S227R (Figure 4B), and 1:1 A129T with Y302N (Figure 4C). Samples were prepared by the urea-dialysis method. The reactions included 1.13 μM [^{32}P]NRI-N~P substrate and 0.3 μM total NRII dimer (heterodimer or control sample) in the presence of 0.3 μM PII (for all three combinations). For comparison the panels also include reactions lacking NRII (showing the intrinsic dephosphorylation rate of NRI-N~P) and containing 0.3 μM each PII and wild-type NRII (show-

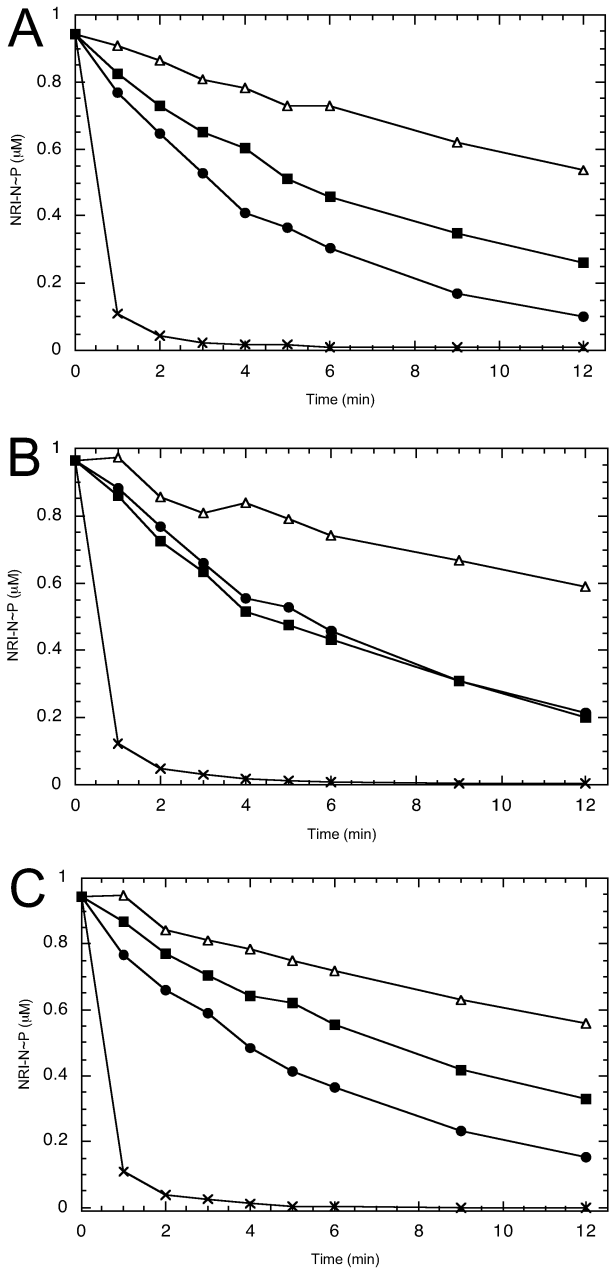

FIGURE 4: Direct phosphatase assay of NRII heterodimers containing single amino acid substitutions in trans. (A) Heterodimer and control samples were prepared by the urea-dialysis procedure using a 1:1 molar ratio of the S227R and Y302N proteins and directly assayed for phosphatase activity (Materials and Methods). The reactions were incubated at 25 °C and contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM AMP-PNP, 50 μM 2-ketoglutarate, 1.13 μM [^{32}P]NRI-N~P, 0.3 μM PII, and the following additions: buffer (△), 0.3 μM control sample (■), 0.3 μM heterodimer sample (●), 0.3 μM wild-type NRII (X). Panels B and C show similar experiments using 1:1 molar ratios of A129T to S227R and A129T to Y302N, respectively. The reaction conditions and symbols are the same as those in panel A.

ing wild-type phosphatase activity). The results showed a pattern consistent with Figure 3 and demonstrated that the S227R::Y302N and A129T::Y302N heterodimers exhibited more phosphatase activity in the presence of PII than the homodimers, while the A129T::S227R heterodimer did not (Figure 4).

We directly examined the autophosphorylation activity of heterodimer and control samples for the S227R with Y302N and A129T with Y302N combinations (Materials and Methods). The reactions contained 1 μM total NRII dimer (heterodimer or control sample) in the absence or presence of 6 μM PII. The S227R::Y302N and A129T::Y302N heterodimer species did not exhibit any restoration of inhibition of autophosphorylation activity in the presence of PII (Supporting Information, Figure 1). Thus, most of the effect observed in Figure 3A,C was probably due to enhancement of the phosphatase activity of the heterodimer species.

The results presented thus far indicated that combining the S227R with Y302N or A129T with Y302N mutant subunits in heterodimers resulted in some restoration of phosphatase activity. The observed complementation could have been due to the presence of two different nonmutant sites in trans in the heterodimer. For example, in the S227R::Y302N heterodimer there is a wild-type PII-binding site trans to the wild-type ATP-lid of the opposing subunit. Alternatively, the complementation could have been due to suppressing effects of a mutation in one subunit on the mutation in the opposing subunit. For example, in the A129T::Y302N heterodimer, the mutant central domain in trans with the mutant ATP-lid may have been the source of the complementation. To explore the two possibilities, we examined the properties of double-mutant NRII homodimers containing two different mutations in each subunit. The behavior of such double-mutant proteins in heterodimer experiments should provide additional information regarding how the domains of NRII collaborate in the phosphatase activity.

Construction and Purification of NRII Double-Mutant Proteins. We constructed plasmids causing the overexpression of the NRII double-mutant proteins, A129T/S227R, S227R/Y302N, and A129T/Y302N. The three proteins were overexpressed and purified to ~ 95% purity (Materials and Methods). All three proteins behaved as dimers on a gel filtration column (data not shown).

Autophosphorylation Activities of the NRII Double-Mutant Proteins. We examined the autophosphorylation activities of the double-mutant homodimers in the absence or presence of PII. Figure 5A shows typical results obtained with wild-type NRII (2 μM) assayed in the absence or presence of 12 μM PII. The inset of panel A shows the results of a qualitative assessment of the autophosphorylation activity (Materials and Methods) and demonstrated that our NRII preparation was essentially completely active. The A129T/S227R and S227R/Y302N proteins autophosphorylated similar to the wild-type protein in the absence of PII and were not inhibited by PII (panels B and C, respectively, of Figure 5). The A129T/Y302N protein exhibited normal autophosphorylation in the absence of PII, and this autophosphorylation was inhibited by PII similar to the wild-type protein (Figure 5D). The preparations of the double-mutant proteins appeared to be completely active, as assessed in qualitative autophosphorylation reactions (insets of Figure 5B−D). The inhibition of autophosphorylation of the A129T/Y302N protein by PII was surprising. We expected this protein to bind PII since a previous study showed that the A129T and Y302N single-mutant proteins exhibited normal PII binding; however, the autophosphorylation of the A129T and Y302N proteins was not significantly inhibited by PII (*40*).

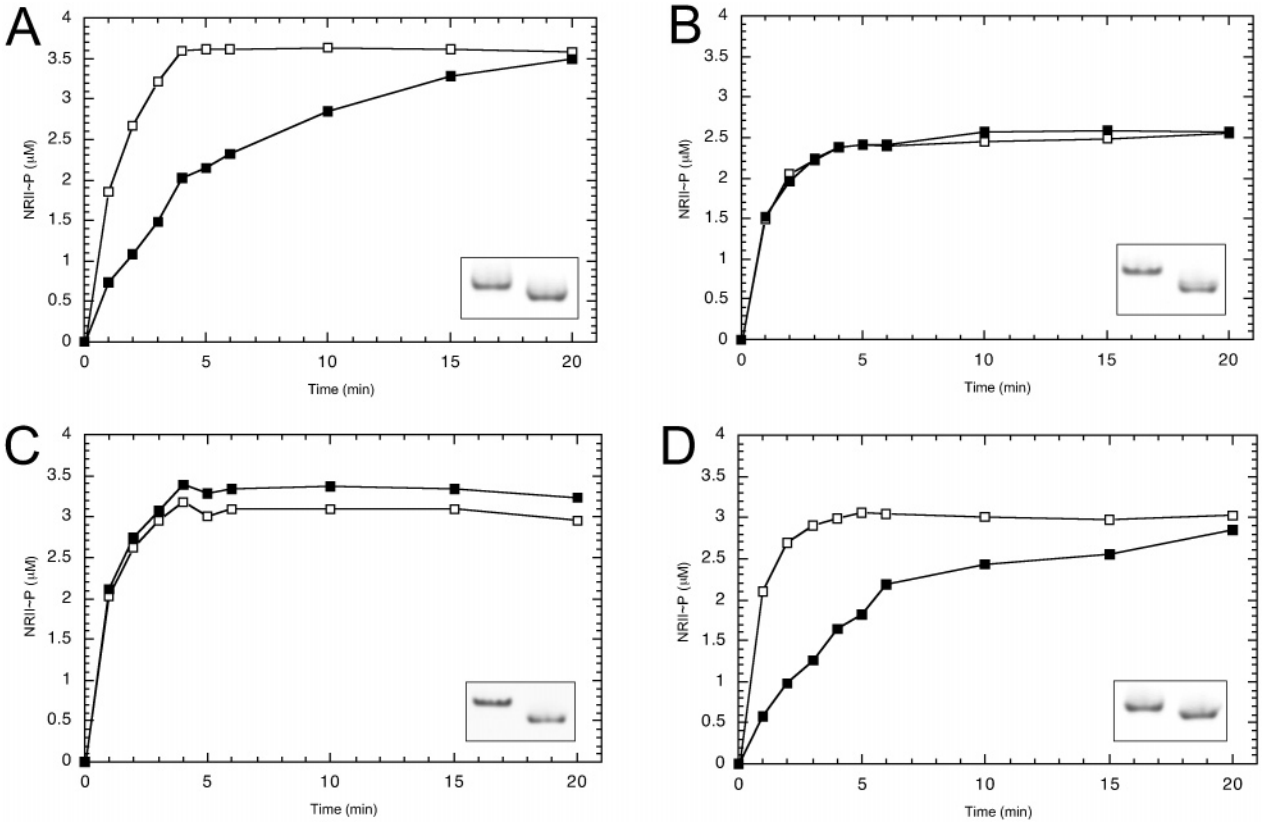

FIGURE 5: Autophosphorylation activities of NRII double-mutant homodimers and their regulation by PII. Wild-type NRII or the indicated double-mutant protein (2 μM dimer) was incubated on ice in reactions containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mg/mL BSA, 0.5 mM [γ-^{32}P]ATP, and 50 μM 2-ketoglutarate in the absence (□) or presence (■) of 12 μM PII trimer. (A) NRII, (B) A129T/S227R, (C) S227R/Y302N, and (D) A129T/Y302N. The insets show a nondenaturing 10% polyacrylamide gel analysis of qualitative autophosphorylation reactions similar to those above, except they lacked BSA, 2-ketoglutarate, and PII and were performed in the absence or presence of unlabeled ATP. These reactions were stopped after 20 min by addition of 50 mM EDTA. Lanes (left to right): no ATP, + ATP.

Kinase and Phosphatase Activities of NRII Double-Mutant Proteins. The balance between the kinase and phosphatase activities of the NRII double-mutant proteins was examined in the combined kinase and phosphatase assay. For each double-mutant protein, we assessed its activities in the absence or presence of excess PII (1 μM) in reactions containing 0.3 μM NRII double-mutant protein, and we compared the activity of the double-mutant protein to the activity of wild-type NRII in an identical reaction. The A129T/S227R and S227R/Y302N proteins exhibited elevated kinase activity in the absence of PII, while in the presence of excess PII (1 μM), these proteins did not exhibit any detectable phosphatase activity (panels A and B, respectively, of Figure 6). A previous study showed that the A129T, S227R, and Y302N single-mutant proteins still retained considerable phosphatase activity when assayed at 1 μM PII (*40*); thus, it seems that combining the S227R mutation with either the A129T or Y302N mutation in a double-mutant protein had an additive effect. The A129T/Y302N protein exhibited elevated kinase activity in the absence of PII, but in contrast to the other double-mutant proteins, it appeared that the A129T/Y302N protein still retained considerable phosphatase activity in the presence of 1 μM PII (Figure 6C). To determine whether this was due to phosphatase activity of the A129T/Y302N protein or due to PII effects on the autophosphorylation of the A129T/Y302N protein, we performed a similar combined kinase and phosphatase assay where PII was omitted from the initial stage of the reaction and once a steady-state level of NRI-N∼P had been reached the reaction was split into tubes containing buffer or various amounts of PII. This method was used previously to assess the phosphatase activities of the L16R, A129T, S227R, and Y302N single-mutant proteins (*40*). The A129T/Y302N protein (0.3 μM) was a very poor phosphatase in the presence of 0.3 μM PII but retained significant phosphatase activity when assayed at elevated PII concentrations (Figure 6D), similar to results obtained previously with the Y302N protein (*40*). Thus, in the A129T/Y302N protein the effects of the single mutations were not additive.

Importantly, for all three double-mutant proteins, no restoration of phosphatase activity was observed. This indicates that the mutations do not complement in cis, and that the combination of two different mutations in trans, in the context of a double-mutant homodimer, does not complement. These results strongly suggest that the mutations do not act as suppressors of each other in trans.

PII-Binding Ability of the NRII Double-Mutant Proteins. Previous results indicated that the S227R mutation resulted in a drastic reduction in PII binding, while the A129T and Y302N mutations had little or no effect on PII binding (*40*). Here, PII binding of the NRII double-mutant proteins was assessed using a cross-linking assay described previously (*23*, *40*). The A129T/Y302N protein was cross-linked to PII in a UV-dependent manner similar to that of the wild-type protein, while both the A129T/S227R and S227R/Y302N proteins exhibited drastically reduced cross-linking to PII,

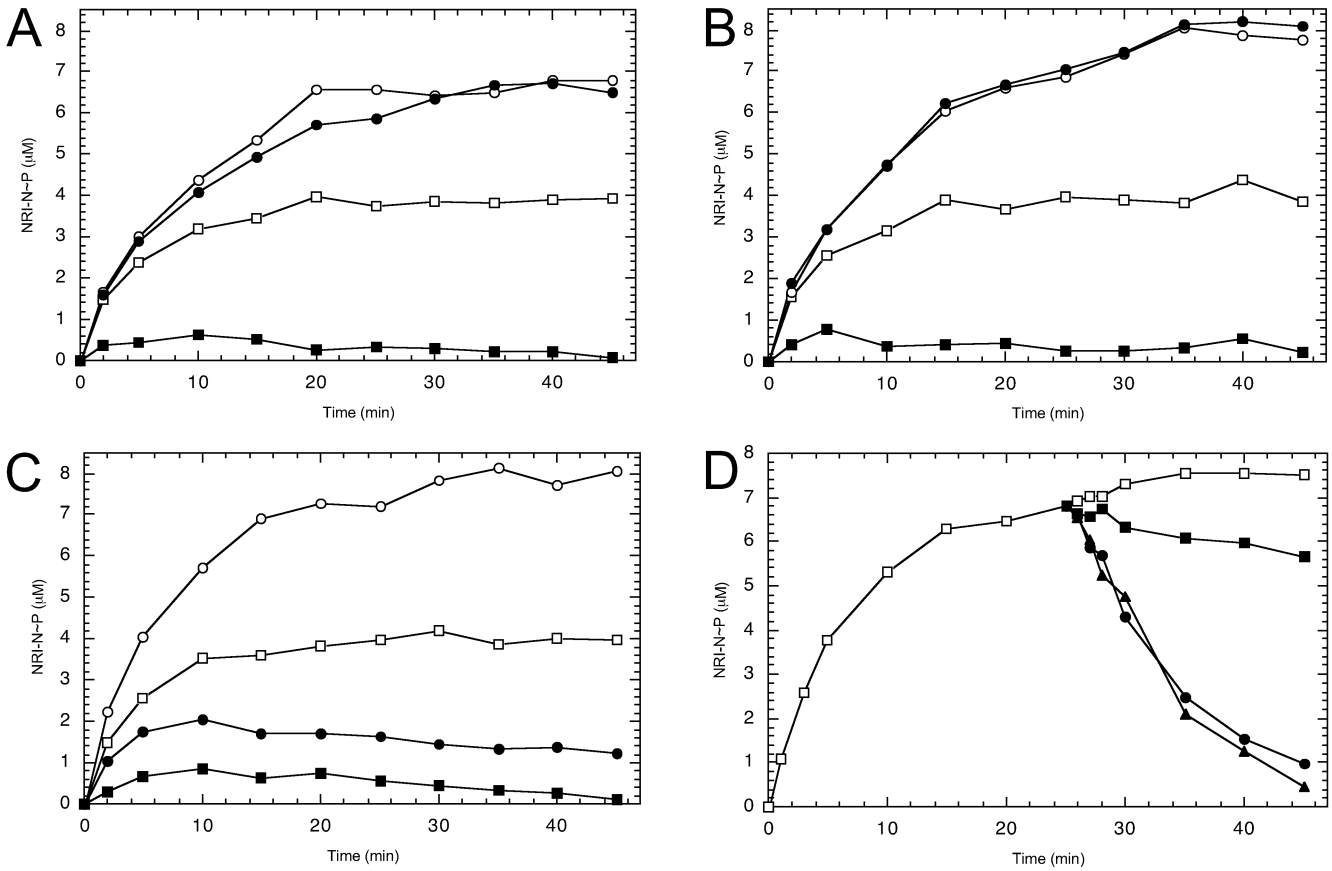

FIGURE 6: Kinase and phosphatase activities of NRII double-mutant homodimers. (A) The A129T/S227R protein or wild-type NRII (0.3 μM) was incubated at 25 °C in the absence or presence of 1 μM PII in reactions containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM [γ-^{32}P]ATP, 50 μM 2-ketoglutarate, and 30 μM NRI-N. Symbols: NRII − PII (□), NRII + PII (■), A129T/S227R − PII (○), A129T/S227R + PII (●). (B) Assay showing the kinase and phosphatase activities of the S227R/Y302N protein. Reaction conditions were the same as those in panel A. Symbols: NRII − PII (□), NRII + PII (■), S227R/Y302N − PII (○), S227R/Y302N + PII (●). (C) The A129T/Y302N protein was assayed with reaction conditions identical to those in panel A. Symbols: NRII − PII (□), NRII + PII (■), A129T/Y302N − PII (○), A129T/Y302N + PII (●). (D) The A129T/Y302N protein (0.3 μM) was incubated at 25 °C in a reaction containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM [γ-^{32}P]ATP, 50 μM 2-ketoglutarate, and 30 μM NRI-N. After 25 min the reaction was split into tubes containing buffer (□), 0.3 μM PII (■), 1 μM PII (●), or 2.7 μM PII (▲).

as expected (Supporting Information, Figure 2). The results presented thus far with the NRII double-mutant proteins suggested that they had all the desired properties for use in heterodimer experiments: they were active kinases, they lacked phosphatase activity or were severely defective for phosphatase activity, their pattern of PII-binding was as expected, and they formed stable heterodimers with MBP-NRII.

Analysis of the Sensitivities of the Combined Kinase and Phosphatase Assay and the Direct Phosphatase Assay. We took advantage of the opportunity afforded by the S227R/Y302N protein to assess the sensitivity of the combined kinase and phosphatase assay in a calibration experiment to determine how much enhancement of phosphatase activity was obtained in the heterodimer experiments. The ability of various amounts of the wild-type NRII protein to counteract the constitutive kinase activity of a fixed level of the S227R/Y302N protein in the presence of excess PII was assessed. The S227R/Y302N protein was ideal for this experiment since it completely lacked the phosphatase activity and did not bind PII. Even though the S227R/Y302N protein formed heterodimers with MBP-NRII (Figure 2), this protein did not form heterodimers very well with wild-type NRII (data not shown); thus, spontaneous subunit exchange is of minimal concern in this experiment. The reactions contained PII in excess (15 μM), the S227R/Y302N protein was 0.3 μM, and wild-type NRII was varied in 3-fold increments from 300 nM down to 1.23 nM. In the presence of 15 μM PII and the absence of competing wild-type NRII, the S227R/Y302N protein exhibited potent kinase activity (Figure 7A). In the absence of the S227R/Y302N protein and the presence of 15 μM PII, wild-type NRII exhibited the expected low steady-state level of NRI-N∼P. In the presence of 0.3 μM S227R/Y302N, 100 nM wild-type NRII was still sufficient to counteract the kinase activity of S227R/Y302N, while 33.3 nM wild-type NRII resulted in an intermediate steady-state level of NRI-N∼P, and 11.1 nM wild-type NRII was not significantly different from the reaction lacking wild-type NRII (Figure 7A). This indicated that the phosphatase activity of wild-type NRII was at least 3-fold more potent than the kinase activity of the S227R/Y302N protein, which itself exhibited elevated kinase activity. By comparing the level of phosphatase activity obtained from 3-fold changes in NRII concentration in this experiment (Figure 7A) with the level of phosphatase activity we observed with our heterodimer samples in Figure 3A,C, we estimate that the complemen-

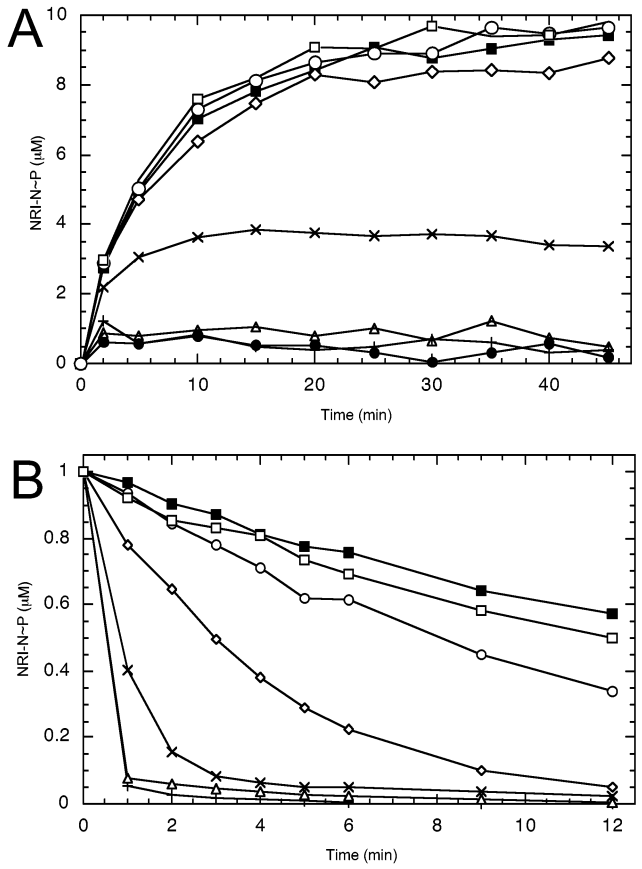

FIGURE 7: Analysis of the sensitivities of the combined kinase and phosphatase assay and the direct phosphatase assay. (A) Effect of varying the level of wild-type NRII in the combined kinase and phosphatase assay in the presence of a fixed level of the S227R/Y302N protein and excess PII. The reactions were incubated at 25 °C and included 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM [γ-^{32}P]ATP, 50 μM 2-ketoglutarate, 30 μM NRI-N, and 15 μM PII, with the following additions: 300 nM S227R/Y302N (■), 300 nM NRII (●), 300 nM each S227R/Y302N and NRII (+), 300 nM S227R/Y302N + 100 nM NRII (Δ), 300 nM S227R/Y302N + 33.3 nM NRII (X), 300 nM S227R/Y302N + 11.1 nM NRII (◇), 300 nM S227R/Y302N + 3.70 nM NRII (○), 300 nM S227R/Y302N + 1.23 nM NRII (□). (B) Effect of varying the level of wild-type NRII in the direct phosphatase assay in the presence of excess PII. The reactions were incubated at 25 °C and included 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM AMP-PNP, 50 μM 2-ketoglutarate, 1.13 μM [^{32}P]NRI-N~P, and 15 μM PII, with the following additions: buffer (■), 300 nM NRII (+), 100 nM NRII (Δ), 33.3 nM NRII (X), 11.1 nM NRII (◇), 3.70 nM NRII (○), 1.23 nM NRII (□).

tation we observed resulted in a 2−3-fold enhancement of phosphatase activity.

A similar experiment was performed to analyze the sensitivity of the direct phosphatase assay. The reactions contained PII in excess (15 μM), and wild-type NRII was varied in 3-fold increments from 300 nM down to 1.23 nM. In the presence of 1.13 μM substrate [^{32}P]NRI-N~P, 33.3 nM wild-type NRII was a potent phosphatase, while 11.1 nM wild-type NRII resulted in an intermediate rate of dephosphorylation, and 3.70 nM wild-type NRII resulted in phosphatase activity similar to the residual phosphatase activities observed with the single mutant proteins assayed at 0.3 μM in the presence of 0.3 μM PII (Figure 7B). The results suggested that the restored phosphatase activity in Figure 4A,C was probably equivalent to a 2−3-fold enhancement, in agreement with the combined kinase and phosphatase assay. The results of the two assays together suggests that the effects observed with the heterodimer samples in the combined kinase and phosphatase assay (Figure 3A,C) were predominantly due to enhancement of phosphatase activity in the presence of PII. These results render unlikely the possibility that the complementation was due to a PII-activated inhibition of phospho transfer from the heterodimer species to NRI-N, or due to a decreased ability of the heterodimer species to bind to NRI-N in the presence of PII.

The Phosphatase Activity of NRII Dimers Requires Two C-Terminal Domains. Since the S227R and Y302N mutant subunits could complement in heterodimers for PII-activated phosphatase activity, we examined the possibility that the two C-terminal domains of the NRII dimer play distinct roles in the phosphatase activity. If so, then the presence of the double-mutant S227R/Y302N subunit in a heterodimer with a wild-type subunit should "kill" the phosphatase activity of the wild-type subunit. For this type of experiment, it is best to have as little of the wild-type NRII homodimer remaining in the heterodimer sample as possible, especially since the phosphatase activity of NRII is much more robust than the kinase activity (Figure 7A). Thus, these experiments were performed by preparing the heterodimer and control samples starting with a 9:1 molar ratio of the double-mutant protein to the wild-type protein. The 9:1 ratio results in a control sample that contains ~10% wild-type homodimer, and this amount of the wild-type homodimer is sufficient to display dramatic phosphatase activity under our assay conditions (Figure 7). In the heterodimer sample, the wild-type NRII homodimer should be reduced to ~1% of the total NRII dimers.

As mentioned earlier, preliminary experiments indicated that the wild-type NRII protein did not form stable heterodimers with the S227R/Y302N protein in appreciable amounts (data not shown). In contrast, the I141V protein formed stable heterodimers with the S227R/Y302N protein in significant amounts (data not shown). Since the I141V protein has wild-type phosphatase activity, this protein was used instead of wild-type NRII. We prepared heterodimer and control samples by the urea-dialysis procedure using a 9:1 molar ratio of the S227R/Y302N protein to the I141V protein and assessed their activities in the combined kinase and phosphatase assay (Figure 8A) and the direct phosphatase assay (Figure 8B). A nondenaturing polyacrylamide gel analysis demonstrated that the heterodimer sample contained the S227R/Y302N::I141V heterodimer and very little I141V homodimer (Figure 8A inset). The heterodimer and control samples (0.3 μM total dimer each) exhibited similar kinase activities in the absence of PII, while the heterodimer sample exhibited significantly less phosphatase activity in the presence of 0.3 μM PII than the control sample (Figure 8A). Similarly, in the direct phosphatase assay, the heterodimer sample displayed dramatically less phosphatase activity than the control sample (Figure 8B).

As a control for the above experiment, we prepared heterodimer and control samples using a 9:1 molar ratio of the A129T/S227R protein to the I141V protein and assessed their activities in the same manner. Figure 8C shows the results of the combined kinase and phosphatase assay. The heterodimer and control samples had similar phosphatase

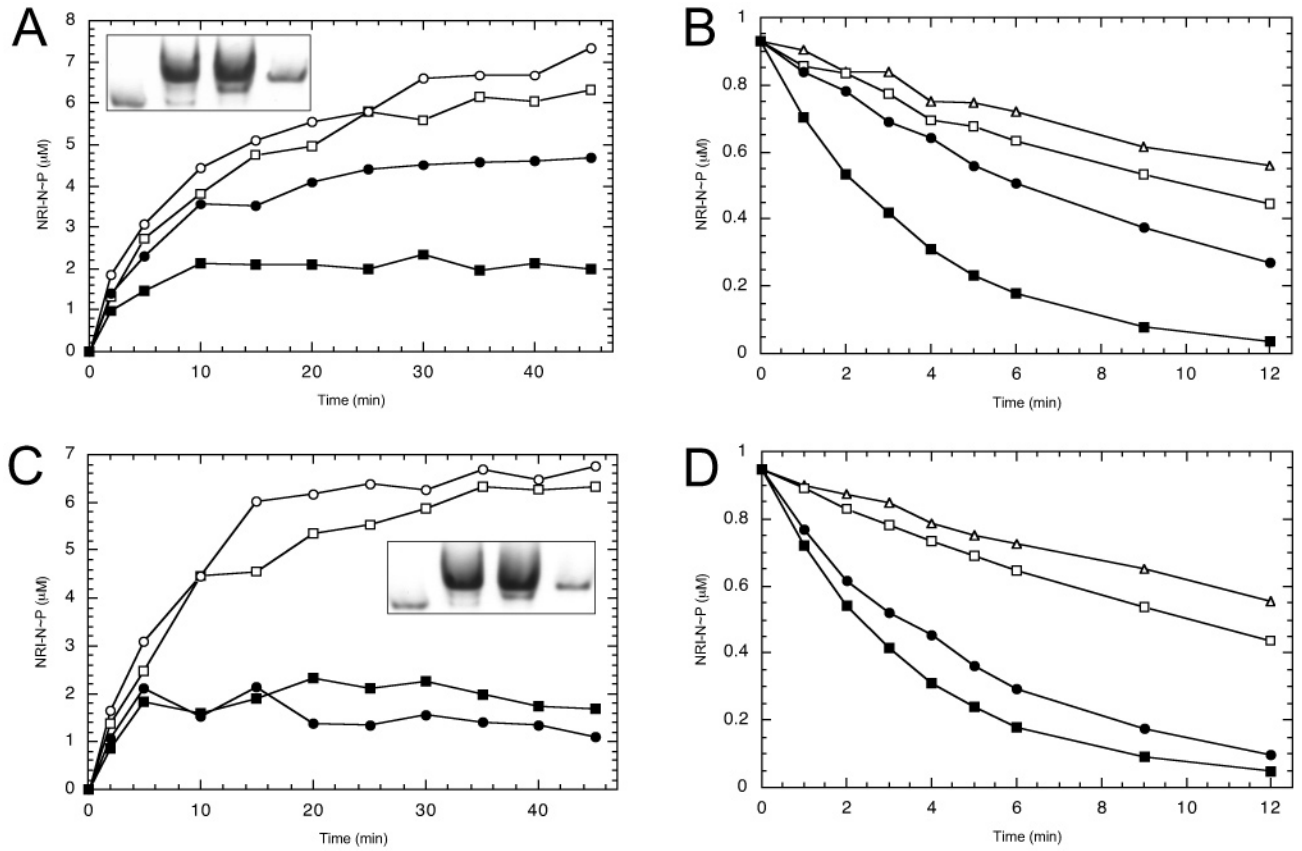

FIGURE 8: The S227R/Y302N protein, but not the A129T/S227R protein, severely diminishes the wild-type phosphatase activity of the I141V protein in heterodimers. (A) Heterodimer and control samples were prepared by the urea-dialysis procedure using a 9:1 molar ratio of the S227R/Y302N protein to the I141V protein (Materials and Methods). The activity of the samples was assessed in the combined kinase and phosphatase assay at 25 °C. The reactions contained 0.3 μM total NRII dimer (control or heterodimer sample) in the absence or presence of 0.3 μM PII and also contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM [γ-^{32}P]-ATP, 50 μM 2-ketoglutarate, and 30 μM NRI-N. Symbols: control sample − PII (□), control sample + PII (■), heterodimer sample − PII (○), heterodimer sample + PII (●). The inset shows a nondenaturing 10% polyacrylamide gel analysis of aliquots of the samples used in the assay. Lanes (left to right): 1 μg of I141V, 5 μg of control sample, 5 μg of heterodimer sample, 1 μg of S227R/Y302N. (B) Heterodimer and control samples were prepared as in panel A, and their phosphatase activity was directly assessed at 25 °C (Materials and Methods). The reactions included 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM AMP-PNP, 50 μM 2-ketoglutarate, and 1.13 μM [^{32}P]NRI-N~P, with the following additions: 0.3 μM PII (Δ), 0.3 μM control sample [no PII] (□), 0.3 μM control sample + 0.3 μM PII (■), 0.3 μM heterodimer sample + 0.3 μM PII (●). (C) Combined kinase and phosphatase assay measuring the activity of heterodimer and control samples prepared by the urea-dialysis procedure starting with a 9:1 molar ratio of the A129T/S227R protein to the I141V protein. The assay conditions are identical to those in panel A. Symbols are the same as those in panel A. The inset shows a nondenaturing 10% polyacrylamide gel analysis of aliquots of the samples used in the assay. Lanes (left to right): 1 μg of I141V, 5 μg of control sample, 5 μg of heterodimer sample, 1 μg of A129T/S227R. (D) Direct phosphatase assay assessing the activity of heterodimer and control samples prepared as in panel C. The assay conditions and symbols are identical to those in panel B.

activities in the presence of 0.3 μM PII (Figure 8C). The direct phosphatase assay revealed a slightly diminished phosphatase activity of the heterodimer sample as compared to the control sample, but it was not dramatic (Figure 8D).

In addition to the combinations highlighted in Figure 8, we also assessed the activities of heterodimer and control samples prepared from a 9:1 molar ratio of the Y302N single mutant protein to the I141V protein. In this case we were unable to sufficiently resolve the heterodimer and homodimer species on nondenaturing polyacrylamide gels to determine unequivocally that the heterodimer species was formed (data not shown). Nonetheless, both the Y302N and I141V proteins were capable of forming heterodimers in multiple other cases, so it is not unreasonable to expect that heterodimer was formed in this case. When assayed in the combined kinase and phosphatase assay, the heterodimer and control samples exhibited similar kinase and phosphatase activities (data not shown).

Intersubunit Complementation of Phosphatase Activity in Heterodimers Containing an A129T/S227R Subunit and a Y302N Subunit. The results presented so far hinted at the possibility that the minimal arrangement of functional sites required for the phosphatase activity would be present within the A129T/S227R::Y302N heterodimer. To test this, heterodimer and control samples were prepared by the urea-dialysis procedure using a 1:1 molar ratio of the A129T/S227R and Y302N proteins, and their activities were assessed in the combined kinase and phosphatase assay (Figure 9A), the direct phosphatase assay (Figure 9B), and the autophosphorylation assay (data not shown). The inset of Figure 9A shows a nondenaturing polyacrylamide gel analysis of aliquots of the samples used in the combined kinase and phosphatase assay. We could not completely resolve the homodimer and heterodimer species for this combination of proteins; however, the gel clearly shows a decrease in the amount of A129T/S227R homodimer in the heterodimer

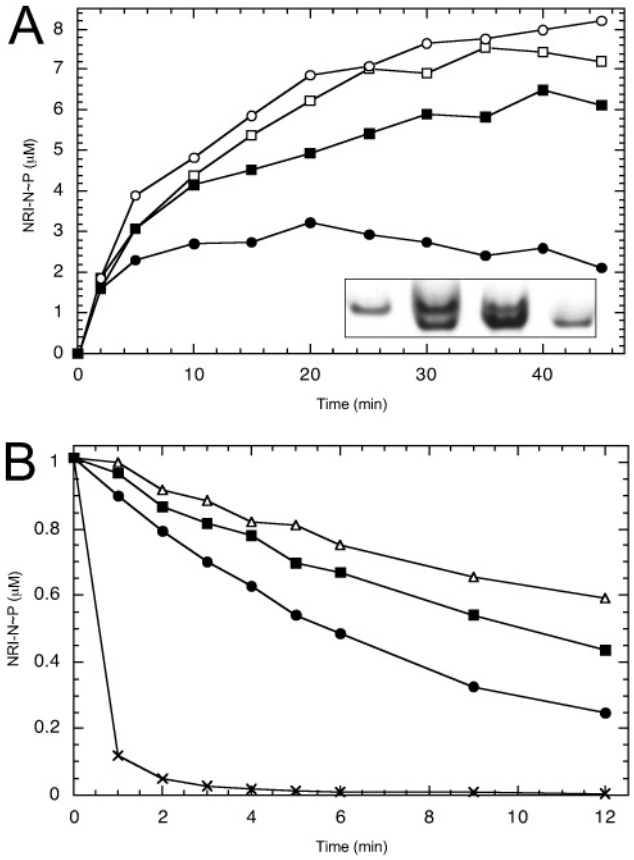

FIGURE 9: Activities of NRII heterodimers containing an A129T/S227R subunit and a Y302N subunit. (A) Combined kinase and phosphatase assay showing partially restored phosphatase activity of the A129T/S227R::Y302N heterodimer. Heterodimer and control samples were prepared by the urea-dialysis procedure using a 1:1 molar ratio of the A129T/S227R and Y302N proteins (Materials and Methods). The activity of the samples was assessed at 25 °C in reactions containing 0.3 μM total NRII dimer (control or heterodimer sample) in the absence or presence of 0.3 μM PII and containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM [γ-^{32}P]ATP, 50 μM 2-ketoglutarate, and 30 μM NRI-N. Symbols: control sample − PII (□), control sample + PII (■), heterodimer sample − PII (○), heterodimer sample + PII (●). The inset shows a nondenaturing 10% polyacrylamide gel analysis of aliquots of the samples used in the assay. Lanes (left to right): 1 μg of A129T/S227R, 3.5 μg of control sample, 3.5 μg of heterodimer sample, 1 μg of Y302N. (B) Direct phosphatase assay showing enhanced activity of the A129T/S227R::Y302N heterodimer. Heterodimer and control samples were formed as in panel A and their activity assessed at 25 °C. The reactions included 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 0.5 mM AMP-PNP, 50 μM 2-ketoglutarate, 1.13 μM [^{32}P]-NRI-N~P, and 0.3 μM PII, with the following additions: buffer (Δ), 0.3 μM control sample (■), 0.3 μM heterodimer sample (●), and 0.3 μM wild-type NRII (X).

sample as compared to that in the control sample suggesting that the heterodimer was formed. The heterodimer sample exhibited enhanced phosphatase activity in the presence of PII, approximately 2−3-fold above the level observed with the control sample (Figure 9A,B). The heterodimer sample did not display restored inhibition of autophosphorylation in the presence of PII as compared to the control sample (data not shown).

Heterodimers Containing an A129T/Y302N Subunit and an S227R Subunit Do Not Exhibit Enhanced Phosphatase Activity. Last, we analyzed heterodimers containing a double-mutant A129T/Y302N subunit and an S227R subunit. We prepared heterodimer and control samples by the urea-dialysis procedure starting with a 1:1 molar ratio of the A129T/Y302N and S227R proteins and assessed their activities in the combined kinase and phosphatase assay and the direct phosphatase assay (Supporting Information, Figure 3). The samples used in the assay had the expected compositions as assessed by a nondenaturing polyacrylamide gel. The combined kinase and phosphatase assay showed that the heterodimer sample had higher kinase activity than the control sample in the absence of PII, but it did not appear to have any enhanced phosphatase activity in the presence of 0.3 μM PII. The direct phosphatase assay showed that the heterodimer and control samples had nearly-identical phosphatase activities in the presence of PII (Supporting Information, Figure 3).

DISCUSSION

The results of this study demonstrate that PII-activated phosphatase activity can be partially restored to phosphatase deficient NRII proteins by including the appropriate mutant subunits in heterodimers. In heterodimers containing the S227R (PII-binding site) and Y302N (ATP-lid) mutant subunits, complementation of phosphatase activity was observed. Since our results with the NRII double-mutant proteins indicated that the complementation was not due to a suppressing effect of one mutation on another, this result suggests that the phosphatase activity requires two NRII C-terminal domains, one from each subunit, and that one provides a functional PII-binding site and the other a functional ATP-lid. This result also shows that a complex of NRII and PII in which PII is bound to a single subunit of the NRII dimer has phosphatase activity. This is consistent with our previous cross-linking study where we showed that the purified cross-linked PII−NRII complex, which had phosphatase activity, consisted of PII covalently linked to a single subunit of the NRII dimer (*23*). The A129T (central domain) and Y302N (ATP-lid) mutant subunits also complemented for phosphatase activity in heterodimers, suggesting that the phosphatase activity requires a functional central domain from one subunit of the dimer and a functional ATP-lid from the opposing subunit. In contrast, the A129T (central domain) and S227R (PII-binding) mutant subunits did not complement for phosphatase activity in heterodimers. All the results taken together suggest a model where the phosphatase activity of NRII requires the collaboration of a functional central domain and PII-binding site in the same NRII subunit, and the ATP-lid of the opposing subunit.

We were unable to determine the NRII N-terminal domain requirement(s) for the phosphatase activity due to the lack of a suitable protein, able to form heterodimers, to use for such studies. Our previous genetic study yielded several mutations in the N-terminal domain of NRII that affected the phosphatase activity; however, only the L16R protein was overexpressed in soluble form and therefore able to be purified (*40*). Our inability to form heterodimers with the L16R protein in this study was apparently not due to an increased stability of the L16R dimer since treatment with 6.7 M urea was not sufficient to allow heterodimer formation. In a previous study, we showed that 6 M urea was sufficient to dissociate the subunits of the L16R dimer (*40*). In addition, we were unable to form heterodimers with truncated derivatives of NRII lacking the N-terminal domain. These results

taken together clearly point to an important role for the N-terminal domain of NRII in dimer stability and heterodimer formation, despite the fact that the isolated N-terminal domain (amino acids 1−110) was monomeric in solution (*38*).

Interestingly, in our heterodimer experiments, the S227R::Y302N and A129T::S227R heterodimer species appeared to have increased kinase activity in the absence of PII, while the A129T::Y302N species did not (Figure 3). One possible explanation for this lies in the properties of the single mutant proteins. The A129T and Y302N proteins both exhibit similar elevated kinase activity, while the S227R protein exhibits wild-type kinase activity (*40*). In the S227R::Y302N and A129T::S227R heterodimer samples, roughly 75% of the total dimers will contain subunits having elevated kinase activity, while in the control samples, only about 50% of the total dimers will contain subunits having elevated kinase activity. This difference could explain the elevated kinase activity observed with these heterodimer samples. In the A129T::Y302N heterodimer sample, the percentage of dimers containing subunits having elevated kinase activity is essentially the same as that in the control sample, and therefore the heterodimer sample did not exhibit significantly increased kinase activity (Figure 3C). This explanation is consistent with all the other experiments. For example, the A129T/S227R::Y302N heterodimer sample did not exhibit increased kinase activity (Figure 9A), while the A129T/Y302N::S227R heterodimer sample did (Supporting Information, Figure 3).

We constructed and purified the NRII double-mutant proteins, A129T/S227R, S227R/Y302N, and A129T/Y302N, to determine the effects of combining the various mutations in a double-mutant homodimer. The A129T/S227R and S227R/Y302N proteins, where one of the mutations reduced PII binding, completely lacked phosphatase activity even when assayed at elevated PII concentrations. The additive effect of these mutations suggests that these pairs of mutations affect distinct functions. In contrast, the A129T/Y302N protein retained a low level of phosphatase activity similar to the Y302N protein (Figure 6). Apparently, deleterious effects in the central domain coupled with mutations in the ATP-lid (at least for A129T and Y302N) are not additive. The A129T and Y302N mutations may affect a common function such that, when combined, the effects are similar to those observed with the more severe of the two mutations, Y302N.

Surprisingly, the A129T/Y302N protein had restored ability to respond to PII with respect to inhibition of its autophosphorylation activity (Figure 5D). This complementation was apparently not due to the combination of the A129T and Y302N mutations in trans since the autophosphorylation of the A129T::Y302N heterodimer sample was not inhibited by PII any more than the control sample (Supporting Information, Figure 1). This suggests that the combination of the A129T and Y302N mutations in cis resulted in the observed PII effect on the A129T/Y302N protein (intra-subunit complementation). However, we cannot rule out that a combination of three or four of the mutant sites in the A129T/Y302N homodimer was required for the PII effect. At this point in time, it is unclear why the two mutations apparently complement in cis for restored inhibition of autophosphorylation.

The double-mutant proteins behaved as predicted by our model when assayed in heterodimer experiments. The wild-type phosphatase activity of the I141V protein was severely diminished by incorporation into heterodimers with the S227R/Y302N protein, but not in heterodimers with the A129T/S227R protein (Figure 8), indicating that the phosphatase activity requires two NRII C-terminal domains. In addition, the A129T/S227R::Y302N heterodimer exhibited restored phosphatase activity (Figure 9), while the A129T/Y302N::S227R heterodimer did not (Supporting Information, Figure 3). Our model predicts that the A129T/S227R::Y302N heterodimer contains the minimal arrangement of wild-type sites necessary for the PII-activated phosphatase activity.

We obtained a 2−3-fold restoration of phosphatase activity from our heterodimer samples in the experiments where we observed complementation. Since this restoration came from ∼50% of the dimers in the heterodimer sample, our results indicate that the heterodimer species exhibited a 4−6-fold restoration of phosphatase activity. This restoration resulted in less phosphatase activity than observed with wild-type NRII (compare Figure 4 and Figure 7B, for example). This could be explained by the possibility that the effect of an individual mutation is not solely limited to the domain it resides in. Indeed, previous studies of truncated derivatives of NRII (*38*), the asymmetry of NRII autophosphorylation (*51*), and mutations affecting the phosphatase activity (*40*) indicated that the domains of the NRII dimer act in a highly concerted manner. Despite its partial nature, the restoration of phosphatase activity was easily discerned in our assay systems.

Previous results have indicated that our in vitro assays are relevant for explaining in vivo observations (*40*) and that a 4−6-fold restoration of the phosphatase activity would be physiologically significant. For example, in intact cells, expression of the nitrogen regulated *glnK* promoter, which is activated by NRI∼P under nitrogen limiting conditions, was about 1.5−3.5-fold higher in cells containing the S227R or Y302N proteins than in cells containing the A129T protein under nitrogen rich conditions (*40*). In addition, cells containing the S227R or Y302N proteins exhibit a severe growth defect on minimal media due to an elevated level of NRI∼P that leads to overexpression of the Nac protein and repression of the *serA* gene (*40*, *52*). Cells containing the A129T protein do not exhibit this growth defect (*39*, *40*). The purified S227R and Y302N proteins exhibited poorer phosphatase activity than the purified A129T protein when assayed in the combined kinase and phosphatase assay (*40*). Thus, both in vivo and in vitro studies indicated that the A129T protein retains more phosphatase activity than the S227R and Y302N proteins. Importantly, the differences in the levels of phosphatase activity observed for the A129T protein versus the S227R and Y302N proteins in the in vitro assay were very similar to the differences in the levels of phosphatase activity observed in this study for the experiments where complementation was observed. It is clear that a 2−3-fold difference in NRII phosphatase activity can have very dramatic consequences in living cells.

Our results have defined how the domains of the conserved transmitter module of NRII collaborate in the phosphatase activity. In addition to the NRII/NRI TCS, transmitter phosphatase activity has been implicated as playing a role in other TCS. For some transmitter proteins, the isolated

central domain has been shown to harbor phosphatase activity (*53*, *54*), similar to NRII. Recently a topological and functional coordination of the central and C-terminal domain of the *E. coli* transmitter protein EnvZ in trans was suggested by Zhu and Inouye (*55*). Unlike NRII, however, the EnvZ protein does not require an accessory protein to activate its phosphatase activity. Certain aspects of our mechanism are likely to apply to other members of the transmitter family of proteins, while the specific mechanism of activation of the phosphatase may differ from protein to protein. The requirement for a functional central domain and ATP-lid in trans may be a conserved feature of the phosphatase activity among the transmitter proteins. It will be interesting to see if the requirement of two C-terminal domains for the phosphatase activity applies to other transmitter proteins or is merely reflective of the need for the accessory PII protein in the case of NRII.

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SUPPORTING INFORMATION AVAILABLE

The autophosphorylation activity of NRII heterodimers containing single amino acid substitutions in trans, in the absence or presence of excess PII; the results of the cross-linking assay that assessed the PII-binding ability of the NRII double-mutant homodimers; the activities of heterodimer and control samples prepared with a 1:1 molar ratio of the A129T/Y302N and S227R proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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