# Homodimerization and hetero-oligomerization of the single-domain trefoil protein pNR-2/pS2 through cysteine 58

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The single-domain human trefoil proteins [pNR-2/pS2 and human intestinal trefoil factor (hITF)] have seven cysteine residues, of which six are involved in maintaining the structure of the trefoil domain. The seventh does not form part of the trefoil domain and is located three residues from the C-terminus. The ability of the pNR-2/pS2 single trefoil domain protein to dimerize was examined by using recombinant protein with either a cysteine or a serine residue at this position by equilibrium ultracentrifugation, laser-assisted desorption MS, gel filtration and PAGE. pNR-2/pS2 $Cys^{58}$ formed dimers, whereas pNR-2/pS2 $Ser^{58}$ did not. Experiments in which the dimer was treated with thiol agents demonstrated that the dimer was linked via a disulphide bond and that the intermolecular disulphide bond was more susceptible to reduction than the intramolecular disulphide bonds. To examine whether dimeric pNR-2/pS2 was secreted by oestrogen-responsive breast cancer cells, which are known to express pNR-2/pS2 mRNA, conditioned medium was separated on non-denaturing polyacrylamide gels, transferred to PVDF membrane and reacted with antiserum against pNR-2/pS2. Monomeric and dimeric pNR-2/pS2 were detected but the majority of the protein reactivity was associated with a larger protein. Treatment of this protein with thiol agents suggested that it is an oligomer containing pNR-2/pS2 linked to another protein by a disulphide bond. These studies suggest that the biological action of pNR-2/pS2 single-domain trefoil protein might involve the formation of homodimers or oligomers with other proteins.

## INTRODUCTION

pNR-2/pS2 is a small cysteine-rich secreted protein [1,2] that is synthesized as an 84-residue precursor from which a 24-residue signal peptide is removed to yield the mature protein of 60 residues. The mRNA encoding the pNR-2/pS2 protein was originally identified by differential screening of breast cancer cells for oestrogen-regulated mRNA species [3–5]. The pNR-2/pS2 mRNA is relatively abundant and is expressed in all oestrogen-responsive breast cancer cell lines [5]. It is not expressed in oestrogen receptor-negative cell lines. pNR-2/pS2 mRNA levels are induced up to 100-fold by oestrogens as a result of an increase in the level of transcription consequent on the interaction of the oestrogen receptor with a non-canonical oestrogen reponse element located 393–405 bp upstream of the transcription start site [6].

The expression of pNR-2/pS2 has been measured in breast tumours in a large number of studies [7–11]. It tends to be co-expressed with the oestrogen receptor [7–9] and is a marker of favourable response to endocrine therapy [8–11], with some reports suggesting that it is a more reliable marker than the oestrogen receptor [8,11].

pNR-2/pS2 is expressed at various levels in normal cells, the highest levels being found in the superficial epithelium of the stomach [12,13]. It is also expressed in the intestine [13] and by normal breast ductal epithelial cells [13]. Ectopic expression of pNR-2/pS2 has been detected in a wide variety of adenocarcinomas including pancreas, large intestine, endometrium, ovary, uterus, bladder and prostate [14].

pNR-2/pS2 belongs to the family of trefoil proteins, characterized by a conserved domain of 41 or 42 amino acid residues. The six conserved cysteine residues (see Figure 1) of the trefoil domain form three intramolecular disulphide bonds and hence three loops that are stacked in a characteristic structure [15–19]. This structure is compact and is relatively resistant to proteases and thiol agents. Mammalian trefoil proteins contain one or two trefoil domains, whereas amphibian trefoil proteins contain up to six trefoil domains, sometimes in association with other protein motifs [1]. There are three known human trefoil proteins: pNR-2/pS2 and human intestinal trefoil factor (hITF) contain one trefoil domain, whereas hSP contains two. The predominant site of expression of all three peptides is in the gastrointestinal tract, pNR-2/pS2 and hSP being expressed mainly in the stomach, and hITF mainly in the duodenum and colon [2,20].

The function of the trefoil peptides is poorly defined. No function for pNR-2/pS2 has been identified in breast tumours or in normal breast tissue. Mice do not normally express the pNR-2/pS2 protein in mammary tissue, and transgenic mice that secrete pNR-2/pS2 from the mammary epithelium during lactation have a normal phenotype [21]. The co-expression of trefoil proteins with mucins [2] and the presence of a mucin-like domain in association with trefoil domains in the integumentary mucin of *Xenopus laevis* skin [22] has prompted the suggestion that they might have a role in the biosynthesis, stability or function of mucus. pNR-2/pS2 'knockout' mice secrete no mucus in the antrum and pyloric region of the stomach, reinforcing the view that pNR-2/pS2 is involved in the formation or maintenance of mucosal integrity [23].

Trefoil peptides are expressed at high levels in sites of ulceration, including the ulcer-associated cell lineage (UACL) in the stomach [2,24], and this has led to suggestions that they might be involved in tissue repair after damage. In the rat, ITF

Abbreviations used: Bistris-propane, 1,3-bis[tris(hydroxylmethyl)methylamino]propane; DTT, dithiothreitol; ITF, intestinal trefoil factor; MEM, minimal essential medium; PSP, pig spasmolytic polypeptide.

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[25] and pNR-2/pS2 [26] protect against indomethacin-induced damage. Thus trefoil peptides might be involved in the surveillance and restitution of the mucosal surface of the gastrointestinal tract. Support for this hypothesis is provided by the demonstration that hSP stimulates the motility of HT29 colonic carcinoma cells [27], hSP and ITF stimulate the motility of non-transformed rat intestinal cells and HT29 cells [28], and transfection of the 410.4 murine adenocarcinoma cell line with pNR-2/pS2 enhances a dispersed growth pattern [29]. ITF knockout mice have an impaired ability to resist the damaging effects of dextran sulphate sodium, suggesting an important role for ITF in the maintenance and repair of the intestinal mucosa [30].

An integral part of the search for trefoil protein function has been attempts to identify proteins or receptors with which they interact. Originally it was reported that the prototype trefoil peptide, pig spasmolytic polypeptide (PSP), interacted with a receptor in rat intestinal epithelial cells [31]. More recently Chinery and Cox [33] reported that after chemical cross-linking of radiolabelled ITF and hSP to solubilized membranes from rat intestine and cultured human breast and colonic cells, ITF and hSP were associated with a protein to form a complex of 45 kDa. Structural studies have identified a possible binding cleft on PSP formed between loops two and three of the trefoil domain [15,16]. A similar structure has been found in pNR-2/pS2; four conserved residues, $Phe^{19}$, $Pro^{20}$, $Pro^{42}$ and $Trp^{45}$, that have solvent-accessible sidechains line this cleft [19]. It has been suggested that these clefts might accomodate either a ribose ring or an aromatic ring of an amino acid during intermolecular interactions.

The extra-trefoil domain cysteine residues of trefoil proteins have attracted interest as possible mediators of covalent intermolecular interactions. Two-domain trefoil proteins such as hSP and PSP contain two extra-trefoil domain cysteine residues that form an intramolecular disulphide bond between the N-terminus and the C-terminus. The single-domain trefoil proteins contain one conserved extra-trefoil domain cysteine residue, three residues from the C-terminus (see Figure 1). The conservation of this free cysteine residue implies that it is of some functional significance. In recombinant ITF this cysteine residue can form intermolecular disulphide bonds to produce dimers [34]. Further, it has been demonstrated that a sample from a patient with Crohn's disease contains hITF and pNR-2/pS2 dimers or heterodimers [34].

We have recently reported the production of recombinant pNR-2/pS2 and the variant protein pNR-2/pS2 $Ser^{58}$ in which the extra-trefoil domain $Cys^{58}$ has been mutated to the isosteric residue serine [35]. The latter recombinant protein has permitted the resolution of the solution NMR structure of pNR-2/pS2 [18,19]. We now report the ability of recombinant pNR-2/pS2 to form homodimers via the formation of an intermolecular disulphide bond between $Cys^{58}$ residues. The susceptibility of this disulphide bond to various thiol agents is characterized and compared with that of the intramolecular disulphide bonds. We demonstrate that pNR-2/pS2 secreted from breast cancer cells also forms dimers and that it can interact via an intermolecular disulphide bond with a second larger protein to form a hetero-oligomer.

## EXPERIMENTAL

### Cell culture

MCF-7 breast cancer cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum and 1 $\mu$g/ml insulin.

### Preparation of conditioned medium

MCF-7 cells were plated in T75 flasks and grown to confluence. They were washed six times with Phenol Red-free minimal essential medium (MEM) with 0.5 h incubations between pairs of washes. The cells were then cultured in MEM supplemented with 1 nM oestradiol for 3 days, after which time the medium was recovered, clarified by centrifugation at 550 ***g*** for 10 min and then frozen at $-20$ °C. The cells were incubated for a further 3 days in MEM supplemented with 1 nM oestradiol, the medium was harvested as before and the cells were discarded. *N*-Ethylmaleimide (10 mM) and iodoacetamide (1 mM) were added to the clarified conditioned medium; it was incubated at 37 °C for 1 h to block free thiol groups and prevent the shuffling of disulphide bonds. The proteins in the conditioned medium were transferred to water by gel filtration and concentrated 100-fold by freeze–drying.

If required, the conditioned medium was treated with cysteine in 20 mM 1,3-bis[tris(hydroxylmethyl)methylamino]propane (Bistris-propane) (pH 6.5)/1 mM EDTA for 18 h at 4 °C.

### Production and purification of recombinant pNR-2/pS2

*Escherichia coli* HB101 transfected with pEZZ18:pNR-2/pS2 $Ser^{58}$ or pEZZ18:pNR-2/pS2 $Cys^{58}$ as described previously [35] were grown to stationary phase overnight in 500 ml of 2 × TY (16 g/l bactotryptone, 10 g/l yeast extract, 5 g/l NaCl) in 2 litre Erlenmeyer flasks. The cells were harvested by centrifugation, washed with 20 % (w/v) sucrose/1 mM EDTA/0.3 M Tris/HCl (pH 8) at room temperature and then shocked osmotically by resuspension in 60 ml of ice-cold 0.5 mM $MgCl_2$ and shaking on ice for 20 min. The extract was clarified by centrifugation at 10000 ***g*** for 10 min at 4 °C, filtered through a 0.2 $\mu$m filter and stored at $-20$ °C.

The recombinant fusion proteins were purified by affinity chromatography on IgG–Sepharose and passed by gel filtration into Factor $X_a$ cleavage buffer. After cleavage for 2–3 days at 4 °C, the recombinant trefoil proteins were purified away from their fusion partners by a second passage over an IgG–Sepharose column as described previously [35].

The recombinant pNR-2/pS2 $Ser^{58}$ protein was purified on a 1 ml Mono Q anion-exchange column in 1-methylpiperazine/HCl, pH 4.5, by elution with NaCl. The protein was then desalted into water by gel filtration and concentrated by freeze–drying.

Dimeric pNR-2/pS2 $Cys^{58}$ was purified as follows. After the second passage over IgG–Sepharose, cysteine was added to 50 mM and the protein was incubated at 4 °C for 24 h. The refolded protein was purified on Mono Q as described above and then concentrated with a Nanospin filter (Gelman) with a 4000 Da cut-off filter. Dimerized protein was purified from residual monomer by gel filtration on Superdex 75 (Pharmacia) in phosphate buffer (50 mM, pH 6.5) and concentrated with a Nanospin filter.

Analytical gel filtration was performed on a 25 ml Superdex 75 column in 20 mM Bistris-propane, pH 6.5, with approx. 5 $\mu$g of protein. The column was calibrated with aprotinin, RNAse A, ovalbumin, BSA and Blue Dextran.

### Analytical ultracentrifugation

Molecular masses were measured by equilibrium ultracentrifugation on a Beckman XLA instrument at 20 °C, with three protein concentrations (1.4, 1.1 and 0.7 mg/ml) and three different rotor speeds. The partial specific volume was calculated from the amino acid composition as 0.706 ml/g and assumed to

be the same in both monomeric and dimeric states. The ideal non-interacting model was adequate to describe all of the data.

### Treatment of recombinant protein

The recombinant pNR-2/pS2 $Cys^{58}$ was treated with cysteine, dithiothreitol (DTT) or glutathione by incubation in 20 mM Bistris-propane (pH 6.5)/1 mM EDTA for 18 h at 4 °C.

### PAGE

The gels shown in Figures 2, 5 and 6 were prepared with the acrylamide ratios and buffers described by Giulian [36]. The separating gels contained 20 % (w/v) and the stacking gels 10 % (w/v) polyacrylamide; both contained 10 % (v/v) glycerol. There was no SDS or reducing agent in any of the buffers used. The proteins were brought to 62.5 mM Tris/HCl (pH 6.8)/12.5 mM EDTA/10 % (v/v) glycerol/0.005 % Bromophenol Blue and were not heated before loading. The gels used for the transfers shown in Figures 7 and 8 were prepared with the acrylamide ratios and buffers advocated by Knowland [37]. The separating gels contained a gradient of 10–35 % (w/v) polyacrylamide and the stacking gels 5 % (w/v) polyacrylamide. There was no SDS or reducing agent in any of the buffers for the transfers shown in Figures 7 and 8(A), but 0.1 % SDS was included in all the buffers of the gel shown in Figure 8(B). For Figures 7 and 8(A) the proteins were treated as described above, but for Figure 8(B), SDS was included in the loading buffer at a final concentration of 2 % (w/v) and the samples were boiled for 5 min before loading. All gels were 10 cm × 8 cm and 0.75 mm thick.

The gels shown in Figures 2, 5 and 6 were stained as described previously [35]. For the experiments shown in Figures 7 and 8, protein was transferred from the gel to 0.2 $\mu$m PVDF membrane (Schleicher and Schuell) by using a semi-dry transfer apparatus (Schleicher and Schuell) for 15 min at 100 mA and then left uncovered at room temperature overnight. They were fixed in 0.2 % glutaraldehyde for 45 min, blocked with 3 % (w/v) BSA in PBS and then incubated with a 1:2000 dilution of rabbit pNR-2/pS2 polyclonal antiserum in 3 % (w/v) BSA and PBS for 2 h at 37 °C. They were incubated for a further 2 h at 37 °C with alkaline-phosphatase-conjugated pig anti-(rabbit IgG) in PBS containing 0.1 % Tween-20 before development with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 30 min at 37 °C. Between incubations the filters were washed three times for 5 min in PBS containing 0.1 % Tween-20. The filters were washed in alkaline phosphatase buffer before addition of the alkaline phosphate substrate.

## RESULTS AND DISCUSSION

### Dimerization of recombinant pNR-2/pS2 $Cys^{58}$

pNR-2/pS2 contains one cysteine residue ($Cys^{58}$) outside the trefoil domain; this is located three residues from the C-terminus (Figure 1) in a relatively unstructured region of the molecule [18,19].

Previous measurements of the molecular mass of an analogue of pNR-2/pS2, in which $Cys^{58}$ had been replaced by serine (pNR-


**Figure 2 Electrophoresis of pNR-2/pS2 $Cys^{58}$ and pNR-2/pS2 $Ser^{58}$ under non-denaturing conditions**

Recombinant pNR-2/pS2 $Cys^{58}$ ($Cys^{58}$) and pNR-2/pS2 $Ser^{58}$ ($Ser^{58}$) were purified, subjected to electrophoresis under non-denaturing conditions and the gels stained as described in the Experimental section.

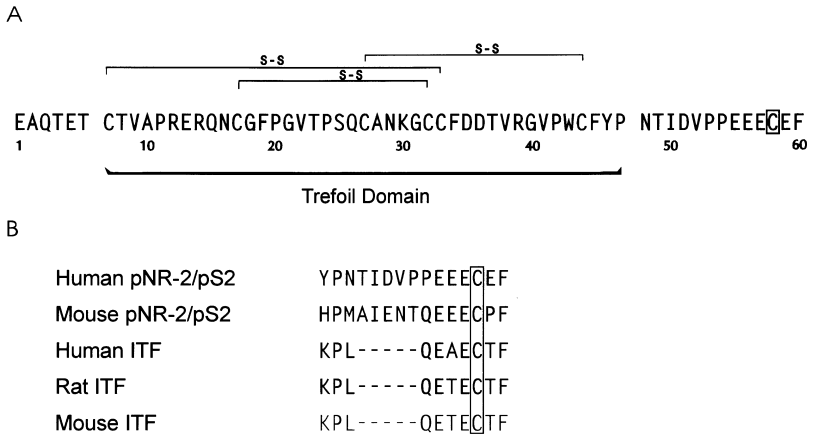


**Figure 1 Sequence of pNR-2/pS2 protein**

(**a**) Sequence of the mature pNR-2/pS2 protein. The positions of the three disulphide bonds and the central forty one amino acids encoding the trefoil domain are marked. The additional cysteine residue at position 58 is boxed. (**b**) The C-termini of human and mouse pNR-2/pS2, and human and rat ITF are aligned and the conserved cysteine residue located three amino acids from the C-terminus is boxed.

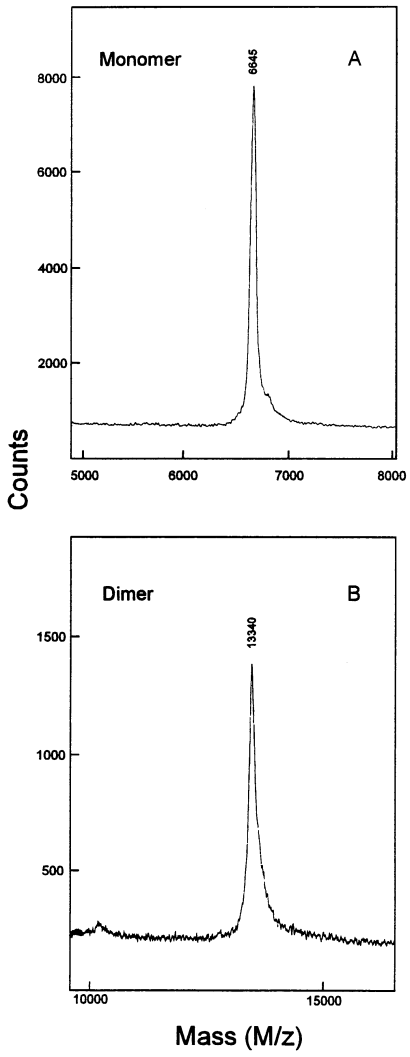


**Figure 3 Matrix-assisted laser desorption MS of pNR-2/pS2 Ser[58] and Cys[58]**

Spectra of pNR-2/pS2 Ser[58] monomer (**a**) and pNR-2/pS2 Cys[58] dimer (**b**). The numbers above the peaks indicate the measured molecular mass.

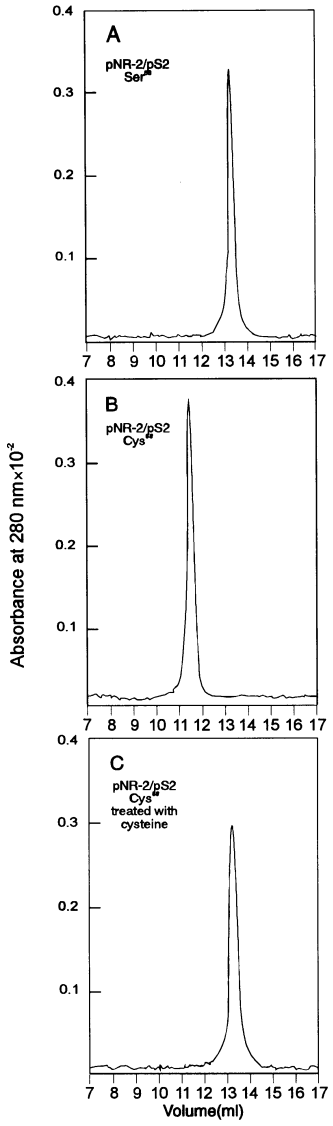


**Figure 4 Gel filtration of pNR-2/pS2 Cys[58] and Ser[58]**

pNR-2/pS2 Ser[58] (5 $\mu$g) (**a**), pNR-2/pS2 Cys[58] (5 $\mu$g) (**b**) or pNR-2/pS2 Cys[58] (5 $\mu$g) (**c**) treated with 400 mM cysteine were analysed by gel filtration on a Pharmacia Superdex 75 gel-filtration column in 50 mM phosphate buffer (pH 6.5)/150 mM NaCl. Absorbance was measured at 280 nm and the elution volumes are shown on the abscissa.

2/pS2 Ser[58]), from the estimates of $^1$H–$^1$H cross-relaxation rates obtained from measurements of the build-up rates for intramolecular nuclear Overhauser effects between the HN-$\epsilon$1 and H-$\delta$1/H-$\zeta$2 protons of Trp[43], gave a molecular mass of pNR-2/pS2 Ser[58] of 6500 Da. This is consistent with this form's being a monomer in solution even at the high protein concentrations used for NMR experiments [19].

Recombinant pNR-2/pS2 Cys[58] and pNR-2/pS2 Ser[58] were subjected to PAGE under non-denaturing conditions without prior treatment with reducing agent (Figure 2). This gel system separates acidic proteins on the basis of their charge and size and showed that pNR-2/pS2 Cys[58] migrated considerably more slowly than pNR-2/pS2 Ser[58]. This suggested that pNR-2/pS2 Cys[58] might form oligomers and that replacement of the cysteine residue by serine abrogated the oligomerization of pNR-2/pS2 Cys[58].

Conditions were then optimized to maximize the formation of the slower-migrating form; the preparation of the highly purified slower-migrating form is described in the Experimental section.

Analytical ultracentrifugation was then used to measure the molecular mass of pNR-2/pS2 Ser[58] and the purified slower-migrating form of pNR-2/pS2 Cys[58] under native conditions in solution. The ideal non-interacting model was adequate to describe all of the data and was consistent with there being no aggregation of the protein at any concentration. The anhydrous molecular mass of the pNR-2/pS2 Ser[58] was $5800 \pm 300$ Da, whereas that of the slower-migrating form of pNR-2/pS2 Cys[58] was $14100 \pm 700$ Da. As the predicted chemical masses of the two forms of the protein are 6674 and 13378 respectively, it is reasonable to conclude that in the native state in solution the two molecules behave as ideal monomers and dimers respectively.

Matrix-assisted laser desorption MS was also used to measure the molecular masses of pNR-2/pS2 Cys[58] and pNR-2/pS2 Ser[58] (Figure 3). The molecular mass of pNR-2/pS2 Ser[58] was measured as 6645 Da, which is very close to the theoretical monomer

**Table 1 Physical characteristics of recombinant pNR-2/pS2 Ser$^{58}$ and Cys$^{58}$**

| Property | pNR-2/pS2 Ser$^{58}$ | pNR-2/pS2 Cys$^{58}$ |
|---|---|---|
| Predicted chemical mass (Da) | 6674 | 13340 |
| Molecular mass by ultracentrifugation (Da) | 5800 ± 300 | 14100 ± 700 |
| Molecular mass by MS (Da) | 6645 | 13378 |
| Stokes radius (Å) | 15 | 20 |
| Frictional coefficient | 1.20 | 1.34 |

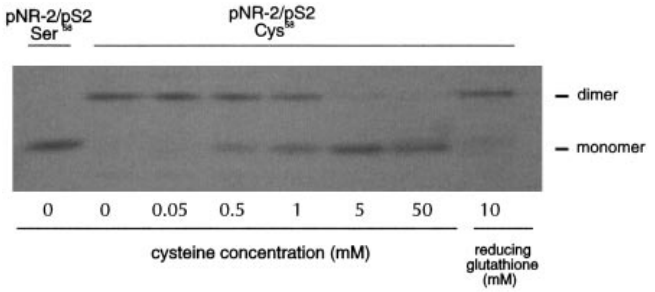


**Figure 5 Effect of cysteine and glutathione on pNR-2/pS2 Cys$^{58}$**

Recombinant pNR-2/pS2 Cys$^{58}$ (2 $\mu$g) was treated with the indicated concentrations of cysteine or reducing glutathione as described in the Experimental section. The samples and untreated pNR-2/pS2 Ser$^{58}$ were analysed on non-denaturing polyacrylamide gels. The positions of monomer and dimer are indicated at the right.

molecular mass of 6674 Da (Figure 3A). The molecular mass of pNR-2/pS2 Cys$^{58}$ was measured as 13340 Da and this is very close to the theoretical molecular mass of 13378 Da for a homodimer (Figure 3B).

The Stokes radii of pNR-2/pS2 Ser$^{58}$ and pNR-2/pS2 Cys$^{58}$ were then measured by gel filtration on Superdex 75 (Figure 4 and Table 1). The pNR-2/pS2 Ser$^{58}$ monomer eluted at 13.1 ml, corresponding to a Stokes radius of 15 Å (1 Å = 0.1 nm), whereas the pNR-2/pS2 Cys$^{58}$ dimer eluted at a volume of 11.4 ml, equivalent to a Stokes radius of 20 Å. The Stokes radius of the pNR-2/pS2 Cys$^{58}$ dimer decreased from 20 to 15 Å after treatment of the purified dimer with cysteine (Figure 4). The Stokes radius was then used to calculate the frictional ratios as described by Siegel and Monty [38], assuming a molecular mass corresponding to the chemical mass (as the molecular mass was consistent with the chemical mass by ultracentrifugation and MS). The frictional ratio was 1.20 for the monomer and 1.34 for the dimer (Table 1), suggesting that neither form of the protein is markedly asymmetric. It is noteworthy, however, that the pNR-2/pS2 Cys$^{58}$ dimer had a larger frictional ratio than the pNR-2/pS2 Ser$^{58}$ monomer, suggesting that dimerization creates a more asymmetric molecule.

To provide evidence that the dimer is formed by a disulphide bond, recombinant pNR-2/pS2 Cys$^{58}$ and pNR-2/pS2 Ser$^{58}$ were treated with various thiol reagents and then subjected to PAGE under non-denaturing conditions. Incubation with increasing concentrations of cysteine converted the slower migrating pNR-2/pS2 Cys$^{58}$ dimer to a form that co-migrated with pNR-2/pS2 Ser$^{58}$ (Figure 5). Incubation with 1 mM cysteine (approx. 30-fold molar excess of cysteine) resulted in the conversion of approx. 50% of the slower-migrating form to the faster-migrating form and 50 mM cysteine (approx. 1500-fold

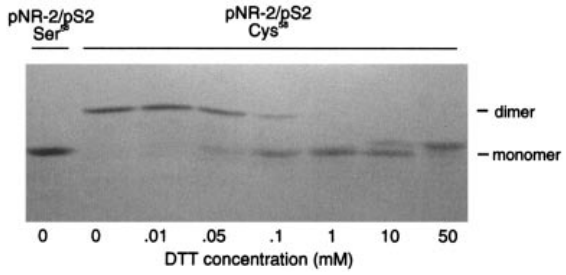


**Figure 6 Effect of DTT on pNR-2/pS2 Cys$^{58}$**

Recombinant pNR-2/pS2 Cys$^{58}$ (2 $\mu$g) was treated with the indicated concentrations of DTT as described in the Experimental section. The samples and untreated pNR-2/pS2 Ser$^{58}$ (2 $\mu$g) were analysed on non denaturing polyacrylamide gels. The positions of monomer and dimer are indicated at the right.

molar excess of cysteine) converted all of the protein to a form that co-migrated with pNR-2/pS2 Ser$^{58}$. In contrast with cysteine, 10 mM reduced glutathione had no effect on the migration of pNR-2/pS2 Cys$^{58}$. DTT had a similar effect to cysteine but it converted pNR-2/pS2 Cys$^{58}$ to the faster-migrating form at lower concentrations (Figure 6), most of the protein being converted to the more rapidly migrating form in the presence of 100 $\mu$M DTT (approx. 3-fold molar excess of DTT). High concentratations of DTT (10 and 50 mM) generated an additional form of pNR-2/pS2 Cys$^{58}$that migrated slightly more slowly than untreated pNR-2/pS2 Ser$^{58}$. On the basis of experiments involving the treatment of pNR-2/pS2 Ser$^{58}$ with DTT we have previously ascribed this slower-migrating form to denaturation of the intramolecular disulphide bonds [35].

The experiments shown in Figures 5 and 6 therefore suggest that pNR-2/pS2 dimerizes through Cys$^{58}$ and that the intermolecular disulphide bond is more suceptible to reduction than the intramolecular disulphide bonds within the trefoil domain.

## Characterization of molecular forms of pNR-2/pS2 secreted from MCF-7 breast cancer cells

The molecular forms of pNR-2/pS2 secreted by MCF-7 breast cancer cells, the cells in which it was first discovered, were then analysed. The proteins in conditioned medium were separated by non-denaturing PAGE and transferred to PVDF; the pNR-2/pS2 protein was then detected with a polyclonal antibody as described in the Experimental section. Figure 7 shows that the conditioned medium contained three distinct forms of pNR-2/pS2, of which two co-migrated with pNR-2/pS2 Ser$^{58}$ monomer and pNR-2/pS2 Cys$^{58}$ dimer. The amounts of monomer and dimer were estimated to be 100 and 10 ng respectively, which corresponds to a concentration of 400 ng/ml monomer and 40 ng/ml dimer in the conditioned medium.

The antisera reacted most strongly with a previously unrecognized third form of pNR-2/pS2 that migrated more slowly than the pNR-2/pS2 dimer (Figure 7). This third form could represent an oligomer in which pNR-2/pS2 is complexed with a second protein; the complex might be stabilized by a disulphide bond involving Cys$^{58}$. To investigate this possibility, concentrated MCF-7-conditioned medium was treated with different concentrations of cysteine before separation by non-denaturing gel electrophoresis, transfer to PVDF and immunodetection with pNR-2/pS2 antiserum (Figure 8A). Cysteine treatment destroyed both the pNR-2/pS2 dimer and the third form of pNR-2/pS2, presumably by reducing the intermolecular disulphide bonds

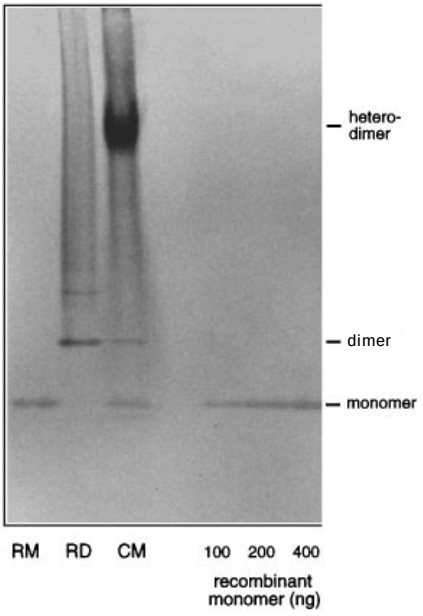


**Figure 7 Western transfer analysis of forms of pNR-2/pS2 protein in medium conditioned by the MCF-7 human breast cancer cell line**

Conditioned medium from the MCF-7 human breast cancer cell line (CM) was subjected to electrophoresis on non-denaturing gels, transferred to PVDF membrane and reacted with pNR-2/pS2 antiserum as described in the Experimental section. Recombinant monomeric pNR-2/pS2 $Ser^{58}$ (RM) and recombinant dimeric pNR-2/pS2 $Cys^{58}$ (RD) were also analysed. The right-hand side of the gel shows different amounts of recombinant monomeric pNR-2/pS2 used to estimate the amount of pNR-2/pS2 in the conditioned medium.

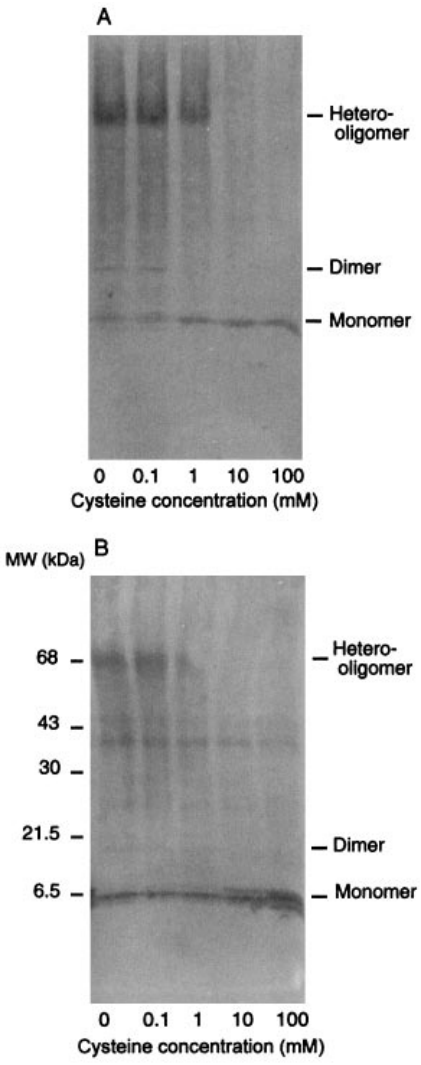


**Figure 8 Effect of cysteine on pNR-2/pS2 heterodimer**

Conditioned medium from the MCF-7 human breast cancer cell line was treated with the indicated concentrations of cysteine, subjected to electrophoresis on non-denaturing (no SDS or 2-mercaptoethanol) (**a**) or non-reducing (SDS but no 2-mercaptoethanol) (**b**) polyacrylamide gels, transferred to PVDF membranes and then reacted with pNR-2/pS2 antiserum as described in the Experimental section. In the presence of SDS the immunodetection of the dimer was decreased; the dimer band is not visible in (**b**). The positions of migration of monomer, homodimer and hetero-oligomer are indicated at the right; the positions of the molecular mass markers (MW) used to estimate the molecular mass of the oligomer are shown at the left in (**b**). The origin or identity of the band of apparent molecular mass 40 kDa is not known.

that stabilized the protein complex. Low concentrations of cysteine (1 and 10 mM) reduced some of the two more slowly migrating forms, whereas only pNR-2/pS2 monomer was detected after treatment with 100 mM cysteine. Both the pNR-2/pS2 dimer and the complex disappeared at approximately the same cysteine concentration, suggesting that the disulphide bonds responsible for the formation of the homodimer and the complex are of similar accessibility and susceptibility to reduction.

The samples of MCF-7 cell conditioned medium were also analysed by gel electrophoresis in the presence of SDS but without reduction to obtain an estimate of the size of the oligomer, as electrophoresis in the presence of SDS is less affected by the charge or conformation of the proteins. The higher-molecular-mass complex migrated with an apparent molecular mass of between 60 and 70 kDa (Figure 8B). The stoichiometry of the oligomer is not known, but if the heteromeric protein comprises one pNR-2/pS2 molecule complexed with one other protein, the size of the other protein would be 53–63 kDa. With this gel system, the sensitivity of detection of the dimer is decreased relative to that of the monomer and the dimer is not readily detected. Samples of MCF-7-conditioned medium that had been treated with different concentrations of cysteine and then separated by this gel system showed that the high-molecular-mass complex is reduced by cysteine and that there is a concomitant increase in the amount of pNR-2/pS2 monomer detected.

The ability of pNR-2/pS2 to form dimers through $Cys^{58}$ might be important in its biological function. $Cys^{58}$ is conserved between pNR-2/pS2 and ITF (Figure 1) and it is noteworthy that the third human trefoil protein, hSP, consists of two trefoil domains whose general structure might resemble that of a dimeric single-trefoil protein. Although the structure of pNR-2/pS2 dimers is not known, there are arguments to suggest that it cannot take up the structure of the prototype two-domain trefoil peptide PSP [19] as has been suggested by Chinery et al. [34] (discussed in [19]). However, it is likely that the patches of conserved residues that are present on one face of each molecule would be in the same orientation in the dimer as in the two-domain molecules.

It has been suggested that the patch of conserved residues, particularly within the cleft between loops 2 and 3, are involved in interactions of trefoil peptides with other proteins such as their receptors or possibly with mucins. One of the roles that has been suggested for trefoil peptides is the biosynthesis and possibly

packaging of mucins; this might involve bivalent binding and require the dimerization of single-trefoil proteins.

Alternatively, $Cys^{58}$ might allow the post-translational covalent interaction of single-domain trefoil peptides to non-trefoil partners, thereby increasing the range of molecules with which trefoil peptides could interact. The identification of a higher-molecular-mass protein in the conditioned medium of MCF-7 breast cancer cell lines that is clearly not a trefoil homodimer might be such a protein. Although the identity of this protein is not known, pNR-2/pS2 is secreted by breast cancer cells and the covalent attachment of pNR-2/pS2 to this protein might be essential for the biological effects of pNR-2/pS2 in breast cancer cells. The identification and characterization of this protein are now a priority.

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