The N-Terminal Cyclophilin-Homologous Domain of a 150-Kilodalton Tumor Recognition Molecule Exhibits Both Peptidylprolyl *cis–trans*-Isomerase and Chaperone Activities†

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ABSTRACT: A cyclophilin-related protein has recently been found to be involved in tumor recognition by natural killer cells. The N-terminal end of this 150-kDa surface molecule (NK-TR) is homologous to cyclophilin/peptidylprolyl *cis–trans*-isomerase. We have constructed a soluble bacterial fusion protein between the cyclophilin-homologous domain of the NK-TR molecule and glutathione *S*-transferase (GST) to test for the presence of peptidylprolyl *cis–trans*-isomerase and chaperone activities and for cyclosporin A binding. The purified NK-cyp-GST fusion protein is shown to accelerate the isomerization of the prolyl peptide bond of the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide with a k_{cat}/K_M value of 7.4×10^5 M^{-1} s^{-1}. The isomerase activity of the NK-TR cyclophilin homolog has been determined to be relatively insensitive to inhibition by the immunosuppressive drug cyclosporin A, with an IC_{50} value of 770 nM as compared to 19 nM for human cyclophilin. Furthermore, the NK-cyp-GST fusion protein has been found to participate in the protein folding process as a chaperone by preventing the aggregation of early folding intermediates of carbonic anhydrase. The implications of the finding of both peptidylprolyl *cis–trans*-isomerase and chaperone activities within the N-terminal domain of a large, cell type-restricted, surface molecule are discussed.

A cyclophilin-related protein has recently been found to be involved in the function of natural killer (NK) cells (Anderson et al., 1993). This 150-kDa surface molecule of human NK cells has been identified as a component of a putative tumor recognition complex (Frey et al., 1991). Cloning and sequencing of the human and mouse cDNAs encoding this NK-TR molecule have revealed that the amino terminus of the predicted protein contains a large hydrophobic region followed by a domain that is highly homologous to the cyclophilin/peptidylprolyl *cis–trans*-isomerase. This cyclophilin-related domain was found to be entirely conserved at the amino acid level between mouse and man. Comparison of the NK-TR cyclophilin-related domain with the amino acid sequences of previously described cyclophilin-related proteins has revealed a 47–53% amino acid identity. Furthermore, greater than 80% of the NK-TR amino acid residues in this region are homologous to one or more of the known cyclophilin proteins.

Proteins of the cyclophilin family display two main properties: they are the intracellular receptors for cyclosporin A (CsA), an immunosuppressive drug, and they exhibit *in vitro* peptidylprolyl *cis–trans*-isomerase activity [reviewed in Stamnes et al. (1992)]. Cyclophilins have been found to be extraordinarily conserved across diverse phyla. They all share a highly conserved core flanked by divergent N- and C-terminal extensions. These extensions appear to serve as signals for targeting and membrane anchoring. Most of the cyclophilins that have been found in multicellular organisms are expressed in a broad range of cells and tissue types. It appears that the highly abundant and widely distributed cyclophilins normally act as "conformases" catalyzing slow steps in the initial folding and/or rearrangement of protein structures (Gething & Sambrook, 1992).

More recently, cyclophilin has been shown to be involved in protein folding not only as an isomerase but also as a chaperone (Freskgård et al., 1992). The chaperone function was shown to prevent *in vitro* the aggregation of an early folding intermediate of denatured carbonic anhydrase whereas the PPIase activity took place later in the folding process.

In the present study, we investigated the N-terminal cyclophilin-homologous domain of a 150-kDa natural killer cell-specific molecule to determine if it exhibits PPIase and chaperone activities as well as CsA binding when expressed as a bacterial fusion protein.

MATERIALS AND METHODS

Preparation and Purification of the NK-cyp-GST Fusion Protein. In order to obtain a soluble NK-TR cyclophilin domain fusion protein, the 5′ hydrophobic region was removed from a cDNA clone encompassing the entire cyclophilin domain by deleting the fragment between the vector *Eco*RI site and the *Xma*III site located at residue 227 (amino acid 60) at the N-terminal end of the cyclophilin homology domain of the NK-TR (Anderson et al., 1993) and religating with the following linker:

AATTCCAGGACC

GGTCCTGGCCGG

The truncated 5′ construct was then cut with *Sma*I (from the pBS-KS vector) and *Sca*I (located at nucleotide 735, amino acid 229, one amino acid away from the C-terminal end of the cyclophilin homology region) and the fragment cloned in

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frame into the *Sma*I site of pGEX-3X (Smith & Johnson, 1988). DH5α-competent bacteria were then transformed with the resulting construct. Individual bacterial clones were tested for production of fusion protein as described in Smith and Johnson (1988) using glutathione–agarose beads (Sigma) and SDS–polyacrylamide gel electrophoresis. A positive clone was selected, and large-scale preparations of NK-cyp-GST fusion protein and of GST were prepared as described in Friedman and Weissman (1991). Purity of the fusion protein preparations was assessed by SDS–polyacrylamide gel electrophoresis. The exact concentration of the proteins was determined by quantitative amino acid analysis using the PITC derivative technique.

Peptidylprolyl cis–trans-Isomerase Assay. The enzymatic activity of the fusion protein was determined using a simplified version of the method described in Kofron et al. (1991). The assay was performed at 5 °C in Tris buffer, pH 7.8, using a Cary 2200 double-beam spectrophotometer interfaced with a computer. The substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma), was dissolved in anhydrous trifluoroethanol (Aldrich) containing 470 mM LiCl. The final *cis* substrate concentration in the assay was 80 μM and was determined as described (Kofron et al., 1991). The data were collected every 0.5 s during typically 3–5 min (i.e., 240–600 points per curve). To simplify the analysis, the reactions were carried out at [S] $\ll K_M$. Under these conditions, the reaction progress curves obey first-order kinetics with a rate constant $k_{obs} = (k_{cat}/K_M)[E]$. Deviations from first-order kinetics were found in certain cases in the early stages of the progress curves due to the initial burst of product resulting from hydrolysis by chymotrypsin of the substrate initially present in the *trans* form. These deviations were detected by plotting the linearized version of the progress curve [i.e., log(Abs) vs time] and were not included in the determination of the rate constants.

Cyclosporin A Inhibition Assay. The enzyme assays were performed as described above except that the samples containing the diluted fusion protein were preincubated for a minimum of 20 min with varying amounts of CsA (kindly provided by Sandoz) (50 mg/mL stock dissolved in Me_2SO). The enzyme and *cis* substrate concentrations in these assays were 80 nM and 80 μM, respectively.

Reactivation of Carbonic Anhydrase. The renaturation of denatured human carbonic anhydrase II (HCA II) was monitored as described in Freskgård et al. (1992). Briefly, HCA II (14 μM) purchased from Sigma was denatured for 1 h or for 10 s at room temperature in 5.0 M guanidine hydrochloride (GuHCl) (Pierce). Reactivation was initiated at room temperature by rapid dilution to 0.3 M GuHCl and 0.83 μM HCA II with 0.1 M Tris-HCl, pH 8.0. The reactivation process was monitored by measuring the CO_2 hydration activity of HCA II (Rickli et al., 1964). The NK-cyp-GST fusion protein was extensively dialyzed against 0.1 M Tris–H_2SO_4, pH 8.0, prior to use in the refolding assays. Purified NK-cyp-GST fusion protein and CsA were used at concentrations of 8.8 and 100 μM, respectively.

RESULTS

A portion of the human NK-TR cDNA encoding amino acids 60–229 (Anderson et al., 1993) was subcloned into the pGEX-3X vector (Smith & Johnson, 1987) for expression of the cyclophilin domain as a soluble NK-cyp-GST fusion protein in *Escherichia coli*. The entire cyclophilin homology region was included in this construct. This fusion protein was then purified to homogeneity using glutathione–agarose beads. The relative molecular weight as determined by SDS–polyacrylamide gel electrophoresis was found to be 45 000 as predicted by the amino acid sequence (data not shown). Exact quantitation of the protein was performed by amino acid analysis of the NK-cyp-GST fusion protein. $A_{280}{}^{1\%}$ was found to be 6.35 cm^{-1}.

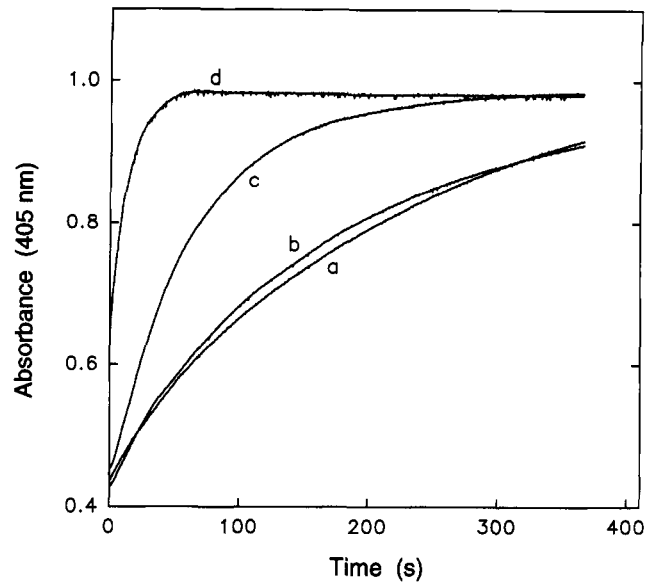

FIGURE 1: Typical progress curves from the chymotrypsin-coupled assay for PPIase activity. (a) Nonenzymatic thermal isomerization with no NK-cyp-GST; (b) 100 nM GST carrier alone; (c) 8 nM NK-cyp-GST; (d) 32 nM NK-cyp-GST.

The NK-cyp-GST fusion protein was then assayed for peptidylprolyl *cis–trans*-isomerase (PPIase) activity using the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. In this coupled assay system conversion of the *cis* Ala-Pro conformer of the substrate to the *trans* form is catalyzed by PPIase. Subsequently, this *trans* form is hydrolyzed specifically by chymotrypsin and this reaction can be monitored at 405 nm. We have used the improved assay conditions described in Kofron et al. (1991). The peptide substrate is dissolved in a LiCl/trifluoroethanol solvent system which improves the signal to noise ratio of the assay by increasing the equilibrium population of the *cis* Ala-Pro conformer from 10% to approximately 50%. Representative progress curves using these assay conditions are illustrated in Figure 1. Addition of increasing amounts of NK-cyp-GST fusion protein clearly accelerates the rate of isomerization of the substrate relative to the uncatalyzed thermal isomerization rate. This effect was not due to the presence of the GST moiety of the fusion protein since the presence of the GST carrier protein in the assay gave a curve similar to that of the uncatalyzed reaction. Variation in the amount of NK-cyp-GST fusion protein generates a linear k_{obs} versus [NK-cyp-GST] plot (data not shown). In the assay conditions used, the *cis* substrate concentration (80 μM) was much lower than the K_M, simplifying the Michaelis–Menten kinetics. By increasing the substrate concentration, the K_M was evaluated to be higher than 400 μM (data not shown). This agrees with the previously published value for the K_M for human cyclophilin of 870 μM (Kofron et al., 1991). The slope of the linear k_{obs} versus [NK-cyp-GST] plot therefore allows us to calculate a k_{cat}/K_M value of 7.4×10^5 M^{-1} s^{-1} for the NK-cyp-GST fusion protein. This value was confirmed by assaying three different preparations of fusion protein.

Cyclosporin A binding to the NK-cyp GST fusion protein was then determined. No binding of [^{3}H]cyclosporin A was detected in the Sephadex LH-20 partition assay (Handschu-

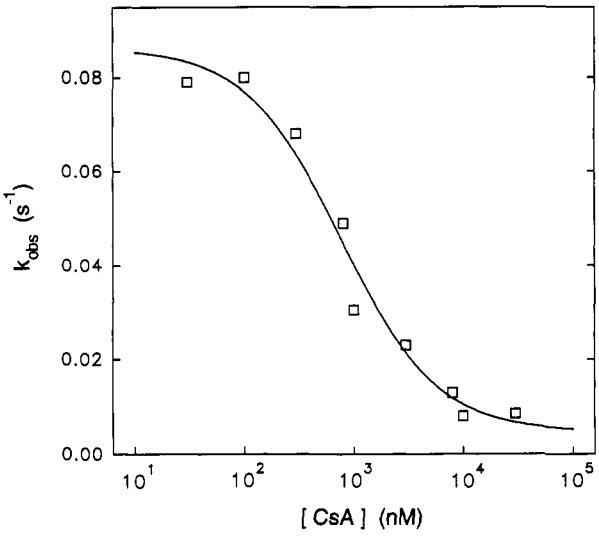

FIGURE 2: Inhibition of PPIase activity of the NK-cyp-GST fusion protein by cyclosporin A. Data were fitted to the following equation: $k_{obs} = k_{obs}^*/(1 + [CsA]/IC_{50})$, where k_{obs}^* is k_{obs} without any inhibitor.

macher et al., 1984), presumably because the low-affinity complexes dissociated during separation on the column (data not shown). The intrinsic tryptophan fluorescence enhancement induced by CsA (Handschumacher et al., 1984) in bovine cyclophilin cannot be detected since the Trp residue involved is not conserved in the NK cyclophilin domain. Therefore, the interaction of CsA with the NK-cyp-GST fusion protein was determined by measuring the inhibition of PPIase activity in the presence of varying concentrations of CsA. Figure 2 shows the results of this experiment. An IC_{50} value of 770 nM has been calculated from this curve.

Since cyclophilin was recently shown to act as a molecular chaperone (Freskgård et al., 1992), the effect of the NK-cyp-GST fusion protein on the reactivation of denatured human carbonic anhydrase (HCA II) was then determined. We found that the NK-cyp-GST fusion protein when added to long-time (1-h) denatured HCA II decreased the $t_{1/2}$ of reactivation from 9 to 4 min and increased the yield of reactivated enzyme from 70% to 100% (Figure 3). These effects were not due to the GST moeity of the fusion protein since GST carrier alone had no effect on the reactivation kinetics (data not shown). These effects are totally abrogated by the addition of CsA. However, if the NK-cyp-GST fusion protein is added at the start of the renaturation and CsA 10 s later, the reactivation is slower than if no CsA is added but 100% renaturation is still achieved. In contrast, if the NK-cyp-GST fusion protein is added 10 s after the onset of renaturation, the $t_{1/2}$ of reactivation is still only 4 min but only 70% reactivation occurs. In order to confirm the chaperone effect of the NK-cyp-GST fusion protein, the effects of the NK-cyp-GST fusion protein on short-time (10-s) denatured HCA were then examined. After only 10 s of denaturation, full denaturation is achieved (Freskgård et al., 1992), but the *cis–trans* isomerization of the peptidylproline bonds should be very limited during the short period of time in the denatured state. The results are shown in Figure 4. The NK-cyp-GST fusion protein did not change the $t_{1/2}$ of reactivation, which remained 1 min, but again increased the yield of active enzyme from 70% to 100%. If both the NK-cyp-GST fusion protein and CsA are added from the onset of renaturation, there is no effect on the reactivation. If NK-cyp-GST fusion protein is added 10 s after the onset of renaturation, the yield of active enzyme is the same as if no fusion protein is added. However, if NK-cyp-GST fusion protein is added from the onset of renaturation and CsA 3 s later, HCA is fully reactivated.

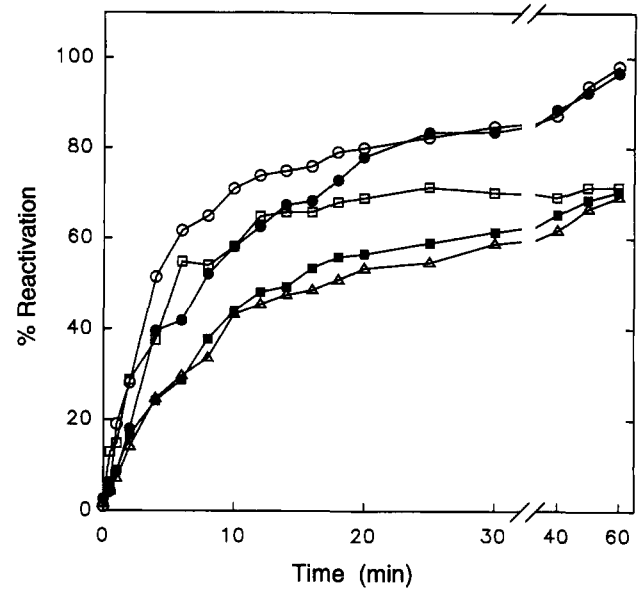

FIGURE 3: Effect of the NK-cyp-GST fusion protein on the time course of reactivation of long-time denatured human carbonic anhydrase II (HCA II). HCA II was denatured for 1 h at room temperature. Renaturation was monitored by measuring the CO_2 hydration activity. (See Materials and Methods for details.) Reactivation of HCA II: (△) no NK-cyp-GST; (○) with NK-cyp-GST; (■) with NK-cyp-GST and CsA; (●) with NK-cyp-GST from the onset of reactivation and CsA added after 10 s; (□) with NK-cyp-GST added 10 s after the onset of reactivation.

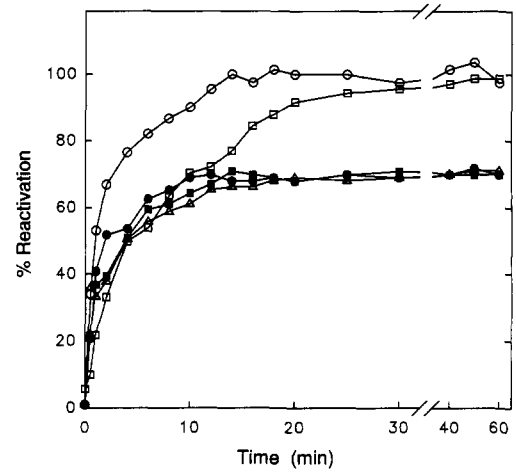

FIGURE 4: Effect of the NK-cyp-GST fusion protein on the time course of reactivation of short-time denatured human carbonic anhydrase II (HCA II). HCA II was denatured for 10 s at room temperature. (See Materials and Methods for details.) Reactivation of HCA II: (△) no NK-cyp-GST; (○) with NK-cyp-GST; (■) with NK-cyp-GST and CsA; (□) with NK-cyp-GST from the onset of reactivation and CsA added after 3 s; (●) with NK-cyp-GST added 10 s after the onset of reactivation.

DISCUSSION

Proteins with PPIase activity are highly abundant and widely distributed in virtually all tissues, in organisms ranging from bacteria to mammals (Stamnes et al., 1992). One family of such proteins, the cyclophilins, have been found to bind the immunosuppressive drug cyclosporin A (Fischer et al., 1989; Takahashi et al., 1989). PPIases accelerate efficiently the *cis–trans* isomerization of prolyl peptide bonds in short oligopeptides (Fischer et al., 1984). The isomerization of

incorrect Xaa–Pro peptide bonds is one of the slow rate-determining steps for *in vitro* refolding of proteins (Brandts et al., 1975; Schmid & Baldwin, 1978). PPIase has been shown to catalyze *in vitro* the refolding of proteins for which it had been suggested previously that proline isomerization was involved in their slow refolding (Lang et al., 1989). However, the real *in vivo* role and physiological substrates of enzymes with PPIase activity remain to be established (Gething & Sambrook, 1992). It seems likely that these proteins would act as conformases catalyzing slow steps in the initial folding and/or rearrangement of protein structures. This hypothesis is supported by the discovery that the gene product of ninaA, a drosophila eye-specific cyclophilin-related membrane protein, is required for the folding of rhodopsins (Stamnes et al., 1991). More recently, PPIases have been shown *in vitro* to be involved in protein folding not only through the isomerization of incorrect peptidylprolyl bonds but also by binding, as molecular chaperones, to early folding intermediates of denatured carbonic anhydrase (Freskgård et al., 1992). Molecular chaperones form a distinct class of molecules involved in assisting protein folding *in vivo*. Chaperone proteins are proposed to function by helping polypeptides to self-assemble by inhibiting alternative assembly pathways that produce nonfunctional structures (Ellis & van der Vies, 1991). PPIases which are also involved in assisting protein folding are not regarded as molecular chaperones since they are thought to stabilize the native state but do not determine the spatial arrangement of the polypeptide backbone (Fischer & Schmid, 1990). The molecular mechanisms of folding assistance by chaperones are unknown, and it is not known whether these proteins are real enzymes. It is postulated that the chaperones offer sites for the reversible binding of partially folded target molecules and thereby suppress aggregation (Fischer & Schmid, 1990).

Recently, a novel cyclophilin homolog has been described (Anderson et al., 1993). This natural killer cell-specific 150-kDa membrane protein was found to contain in its N-terminal region a domain with considerable homology to cyclophilins. In order to determine if this cyclophilin-like domain indeed possessed PPIase activity as predicted by its amino acid sequence, we produced a bacterial fusion protein between this region and glutathione *S*-transferase (GST). This NK-cyp-GST fusion protein was found to accelerate the isomerization of the prolyl peptide bond in the tetrapeptide substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Figure 1). A k_{cat}/K_M value of 7.4×10^5 M^{-1} s^{-1} was obtained at 5 °C for the NK-cyp-GST fusion protein. k_{cat}/K_M values determined in similar assay conditions have been reported in the range of 1×10^7 M^{-1} s^{-1} at 10 °C for human cyclophilin A (Price et al., 1991; Liu et al., 1990) and for *E. coli* cyclophilin (Liu & Walsh, 1990). Kofron et al. (1991) report a value of 1.46×10^7 M^{-1} s^{-1} in assays performed at 0 °C. A value of 1.9×10^6 M^{-1} s^{-1} at 10 °C has been reported for Cyp-40, a 40-kDa cyclophilin-related protein (Kieffer et al., 1992). The NK-cyp-GST fusion protein therefore appears to possess a somewhat lower but still significant activity than most of the other cyclophilin homologs in the *in vitro* assay conditions for PPIase activity using a defined synthetic tetrapeptide as a substrate. It has been demonstrated that the substrate specificity of human cyclophilin shows little dependence on the Xaa residue when a series of peptides with the general structure *N*-succinyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide were tested (Harrison & Stein, 1990). However, another report suggests that the more sequence-divergent members of the cyclophilin family may show intracellular substrate or ligand discrimination (Bergsma et al., 1991). Most of the residues thought to be involved in the PPIase activity of cyclophilin are conserved in the NK-TR cyclophilin domain (Figure 5), suggesting a conserved mechanism. However, it is possible that the substrate specificity of the NK-TR cyclophilin domain is not identical, perhaps reflecting different *in vivo* substrates. It is also conceivable that the lower k_{cat}/K_M observed for the NK-cyp-GST fusion protein as compared to those of most of the other cyclophilin homologs might be due to the absence in the fusion protein of a structural component present in the native NK-TR molecule.

The effect of the immunosuppressive drug cyclosporin A (CsA) on the PPIase activity of the NK-cyp-GST fusion protein was also determined. The IC_{50} value was found to be 770 nM. The reported IC_{50} value for human cyclophilin A is 19 nM (Liu et al., 1990). The affinity of the NK-cyp-GST fusion protein for CsA is much lower than that of its human cyclophilin A homolog. In this respect, the NK-TR cyclophilin domain is more similar to the *E. coli* cyclophilin, which has a reported IC_{50} value of 3000 nM (Liu et al., 1991). Indeed, the sensitivity to inhibition by CsA has been correlated with a specific tryptophan residue (Liu et al., 1991) which is absent from both *E. coli* and NK-TR cyclophilins (Figure 5).

The chaperone activity of the NK-cyp-GST fusion protein was determined on the basis of its effect on the refolding of denatured human carbonic anhydrase. The presence of NK-cyp-GST fusion protein reduced the $t_{1/2}$ of the reactivation from 9 to 4 min by accelerating the folding of carbonic anhydrase (which contains 15 *trans*- and 2 *cis*-proline residues). This confirms the presence of PPIase activity in the NK-cyp-GST fusion protein. The fact that the yield of active carbonic anhydrase increases from 70% to 100% when the NK-cyp-GST-fusion protein is present during the refolding process indicates that the NK-TR cyclophilin domain also acts as a molecular chaperone. The NK-cyp-GST fusion protein has independent effects on the kinetics and the yield of active enzyme. This result confirms the presence of a chaperone activity in the fusion protein. This is illustrated in Figure 3 since after 10 s of refolding, inhibition of the PPIase activity by CsA still yields 100% activity with a relatively slow $t_{1/2}$ (8 min), whereas addition of the NK-cyp-GST fusion protein after 10 s of renaturation decreases the $t_{1/2}$ to 5 min while the yield is not increased. Examination of the results for short-time (10-s) denatured HCA II (Figure 4) also confirms the chaperone activity of the NK-cyp-GST fusion protein. Indeed, during the short denaturation period very little isomerization of the peptide bonds is likely to occur, but, nevertheless, addition of the NK-cyp-GST fusion protein increases the yield of active HCA II from 70% to 100% without modifying the kinetics of reactivation. Cyclophilin is postulated to act as a molecular chaperone by binding to an early folding intermediate and thereby preventing aggregation of this intermediate (Freskgård et al., 1992). The NK-cyp-GST fusion protein appears to exert its chaperone activity by the same mechanism as its cyclophilin A homolog since both proteins yielded similar results when tested in the refolding of denatured carbonic anhydrase.

We have shown that the N-terminal domain of NK-TR, a large surface molecule which is thought to be involved in tumor recognition by natural killer cells, exhibits both PPIase and chaperone activities. The expression of this molecule was found to be restricted to NK cells and to be expressed at low levels in these same cells (Frey et al., 1991; Anderson et al., 1993). The NK-TR cyclophilin domain differs therefore from

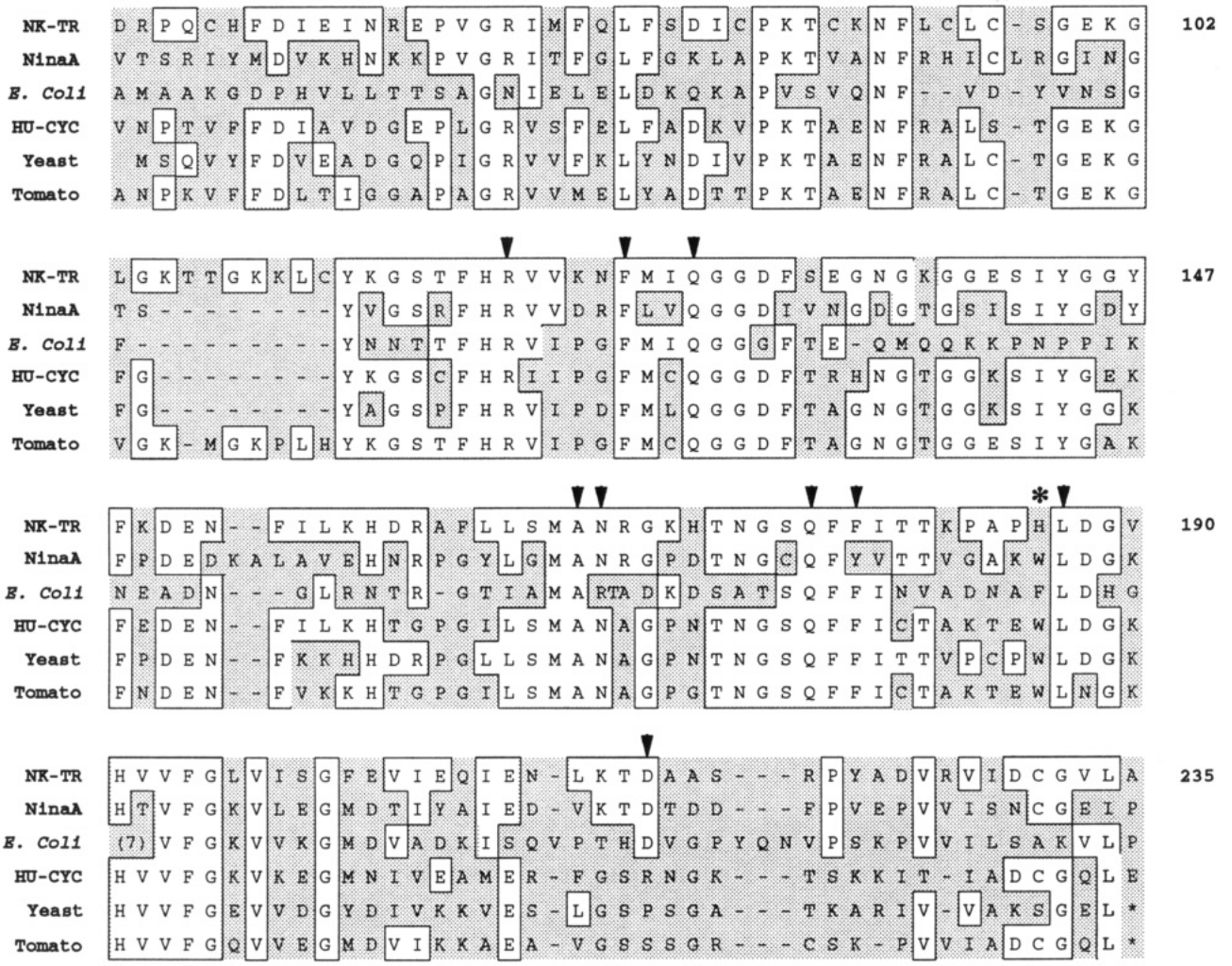

FIGURE 5: Amino acid sequence alignment of several cyclophilin homologs to the N-terminal domain of the NK-TR. Numbering is according to the NK-TR sequence from Anderson et al. (1993). The residues of the human cyclophilin active site are indicated by arrowheads (Kallen & Walkinshaw, 1992). All but one of these residues are conserved in the NK cyclophilin domain homologs, and the differing residue is contained in a C-terminal region that is very poorly conserved among the various cyclophilins. This diverging region could be involved in defining substrate specificity. The tryptophan residue (indicated by an asterisk) of human cyclophilin that has been shown to be important for CsA binding is not conserved in either the NK or the *E. coli* homologs, and this correlates with the decreased CsA binding affinity of these two molecules (Liu et al., 1991).

most of the other more ubiquitous cyclophilin homologs by the fact that it is a domain of a large molecule and that it is expressed at low levels in a very restricted cell type. One question that arises is what might be the *in vivo* substrate for the NK-TR cyclophilin domain. Perhaps, it is involved, like the drosophila ninaA cyclophilin homolog, in assisting in the proper folding of an NK-restricted protein. Fischer and Schmid (1990) have also proposed that PPIases could be involved in the recognition and/or interconversion of certain surface-exposed turns of folded proteins that contain proline residues. Another possibility is that, since the cyclophilin-homologous region is at the amino-terminal end of the NK-TR protein, it is involved in the proper folding of the rest of the molecule as it is synthesized. Examination of the NK-TR amino acid sequence reveals several proline-rich regions, one of which immediately follows the cyclophilin domain. The PPIase activity of the cyclophilin domain could accelerate the intrinsically slow isomerization of incorrect prolyl peptide bonds in these proline-rich regions, thereby decreasing the risk of proteolytic degradation of partially folded chains while the chaperone activity would suppress competing processes such as aggregation (Fischer & Schmid, 1990).

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