Expression and Mutagenesis of the Novel Serpin Endopin 2 Demonstrates a Requirement for Cysteine-374 for Dithiothreitol-Sensitive Inhibition of Elastase†

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ABSTRACT: The primary sequence of the serpin endopin 2 predicts a reactive site loop (RSL) region that possesses high homology to bovine elastase inhibitor, suggesting inhibition of elastase. Moreover, endopin 2 possesses two cysteine residues that implicate roles for reduced Cys residue(s) for inhibitory activity. To test these predicted properties, mutagenesis and chemical modification of recombinant endopin 2 were performed to examine the influence of dithiothreitol (DTT), a reducing agent, on endopin 2 activity. Endopin 2 inhibited elastase in a DTT-dependent manner, with enhanced inhibition in the presence of DTT. The stoichiometry of inhibition in the presence of DTT occurred at a molar ratio of endopin 2 to elastase of 8/1, resulting in complete inhibition of elastase. However, a higher molar ratio (25/1) was required in the absence of DTT. DTT enhanced the formation of SDS-stable complexes of endopin 2 and elastase, a characteristic property of serpins. Site-directed mutagenesis of endopin 2, with substitution of Ala for Cys-232 or Cys-374, demonstrated that Cys-374 (but not Cys-232) was required for the DTT-sensitive nature of endopin 2. Chemical modification of Cys-374 by bis(maleimido)ethane also reduced inhibitory activity. Modified electrophoretic mobilities of mutant endopin 2 suggested the presence of intramolecular disulfide bonds; in addition, chemical modification suggested that Cys-374 influences the electrophoretic and conformational properties of endopin 2. Moreover, the reducing agent glutathione enhanced endopin 2 activity, suggesting that glutathione can function as an endogenous reducing agent for endopin 2 in vivo. These findings demonstrate the importance of Cys-374 for DTT-sensitive inhibition of elastase by endopin 2.

Serpins represent a diverse family of endogenous mammalian protease inhibitors that participate in the control of cellular and physiological functions (*1*, *2*). We recently cloned two novel bovine serpin cDNAs known as endopin 1 and endopin 2, from adrenal medulla and liver, respectively, that possess high homology (70%) to one another (*3*, *4*). The homology of the cloned bovine liver α_1-antichymotrypsin-like (ACT-like) serpin to endopin 1 provides the basis for referring to this bovine liver serpin as endopin 2 in this study. Endopin 1 mRNA is expressed in neuroendocrine tissues (adrenal medulla, pituitary, and pancreas) and not in liver (*4*, *5*). The endopin 2 serpin, however, displays a more widespread distribution since it is expressed in neuroendocrine (adrenal medulla, pituitary, and pancreas) and non-neuroendocrine (liver) tissues (*3*, *5*).

Endopin 1 and endopin 2 possess reactive site loop (RSL) domains that are known to characteristically influence the target protease specificities of serpins. The distinct primary sequences of the reactive site loop (RSL) domain of endopin 1 and 2 suggest that different target proteases are inhibited by these serpins. On the basis of alignments with related serpins, the RSL domain of endopin 1 possesses a predicted P1−P1′ site of Arg−Thr. The P1−P1′ residues of a serpin often mimic the cleavage specificity of the target protease. Therefore, endopin 1 may inhibit proteases cleaving at Arg or basic residues such as trypsin. Indeed, endopin 1 effectively inhibits trypsin (*4*). Although endopin 1 possesses homology to ACT, chymotrypsin was not inhibited.

The RSL of endopin 2 differs from that of endopin 1. Primary sequence comparisons with other serpins indicates that the RSL of endopin 2 has highest homology with the bovine elastase inhibitor (*6*). Alignment of the RSL domains of endopin 2 with bovine elastase inhibitor (*6*), rabbit α_1-antiproteinase inhibitor (*7*), and related serpins (*4*, *5*, *8*−*10*) suggest that the P1−P1′ site of endopin 2 may be located at the Ser−Ser peptide bond within its RSL region. These homologous RSL domains strongly suggest that endopin 2 may inhibit elastase. The primary sequence of endopin 2 also indicates two Cys residues at positions 232 and 374 whose state of oxidation or reduction could influence endopin 2 inhibitory activity. Possible roles for Cys residues in the function of antithrombin, α_1-antitrypsin, or other serpins have been suggested (*11*) but have not been demonstrated. Therefore, roles for reduced or oxidized Cys residues in modifying the inhibitory activity of endopin 2 should be evaluated.

To test the predicted properties of endopin 2 based on its primary sequence in this study, recombinant NH_2-His-tagged endopin 2 was expressed to examine DTT-sensitive inhibition of elastase by endopin 2. The role of Cys-232 or Cys-374 for the inhibitory activity of endopin 2 was assessed by site-directed mutagenesis and chemical modification of sulfhydryl

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groups by BMOE [bis(maleimido)ethane]. Results indicated that Cys-374, but not Cys-232, was responsible for the DTT-sensitive activity of endopin 2. Moreover, altered electrophoretic mobility of endopin 2 by mutagenesis or chemical modification of Cys residues suggested that Cys-374 influences the conformation of endopin 2. Results also indicated that glutathione, a cellular reducing agent in vivo, can serve as reducing agent for endopin 2 activity. These results demonstrate the important role of Cys-374 for the DTT-dependent inhibitory activity of the serpin endopin 2.

MATERIALS AND METHODS

Expression and Purification of Wild-Type and Mutant Forms of Recombinant Endopin 2. Wild-type and mutant forms of endopin 2 were each expressed in *Escherichia coli* by use of the pET19b plasmid vector (Novagen, Madison, WI), with an NH_2-His tag consisting of $MG(H)_{10}SSKHI-(D)_4KHM$, which allows affinity purification on a Ni^{2+} column, as described previously (*4*). Site-directed mutagenesis was utilized to generate endopin 2 mutants that substituted Ala for Cys-232 or Cys-374 (single-Cys mutants), and substituted Ala for both Cys-232 and Cys-374 (double-Cys mutant).

Substitution of Ala for Cys-232 was achieved by PCR site-directed mutagenesis to generate the C232A mutant. PCR generated a partial cDNA fragment corresponding to the NH_2-terminal domain of endopin 2 that substituted Ala for Cys-232 by use of primers a and b; a second PCR reaction with primers c and d generated a partial cDNA fragment corresponding to the COOH-terminal domain of endopin 2. Primers a, b, c, and d consisted of 5′-AAAAAAACATATGCTCCCAGAGAATGTGAC-3′ (with incorporation of an *Nde*I site, underlined), 5′-GGCGCCCAGCTCCTCGTCCC-3′ (that includes the Ala substitution, underlined), 5′-ACGCTGGTGGAGCTCACATACA-3′, and 5′-AAAAACTCGAGCTAG-GCTTCCTTGG-3′ (with incorporation of a *Xho*I site, underlined), respectively. These PCR reactions utilized 50 ng of endopin 2 cDNA template (*3*), 0.4 μM primers, and Pfu DNA polymerase (10 units, Stratagene, La Jolla, CA), with 25 thermocycles, each consisting of 94 °C for 50 s, 44 °C for 1 min, and 72 °C for 1 min. PCR products were then phosphorylated with T4 kinase and ATP, as described by the manufacturer (Gibco Life Technologies). The resultant NH_2-terminal domain cDNA fragment of 713 bp was digested with *Nde*I, and the COOH-terminal domain cDNA fragment of 478 bp was digested with *Xho*I. These two PCR-generated fragments were joined by blunt-end ligation and ligated to the pET19b vector at *Nde*I and *Xho*I sites. The resultant C232A mutant cDNA was subjected to DNA sequencing [as described previously (*3*, *4*)] to ensure that the Ala substitution was obtained, and that the endopin 2 cDNA sequence was correctly amplified by PCR.

The C374A mutant form of endopin 2 was generated similarly by PCR amplification of partial cDNA fragments representing NH_2- and COOH-terminal domains, with incorporation of an Ala substitution for Cys-374. PCR of the partial cDNA representing the NH_2-terminal domain utilized primers consisting of 5′-AAAAAAACATATGCTCCCAGAGAATGTGAC-3′ (primer a), which incorporated an *Nde*I site (underlined), and 5′-GAATATGGAAAGCAGAAAGGGCCTGTT-3′ (primer e). PCR of the partial cDNA representing the COOH-terminal domain utilized the primers 5′-pGCCAAAGAGACTCAGAGCATCATCTTTTTG-3′ (primer f), which included the Ala substitution (underlined), and 5′-AAAAACTCGAGCTAGGCTTCCTTGG-3′ (primer d), which incorporated a *Xho*I site (underlined). PCR was performed under the same reaction conditions as described for the C232A mutant (previous paragraph). PCR products representing partial cDNAs for NH_2- and COOH-terminal domains of endopin 2 were phosphorylated with T4 kinase and ATP, digested with *Nde*I and *Xho*I, respectively, and ligated by blunt-end ligation. The C374A mutant cDNA was ligated to the pET19b vector at *Nde*I and *Xho*I sites; DNA sequencing confirmed that the mutation was obtained.

The double mutant with Ala substitutions for Cys at positions 232 and 374 was generated from the C232A and C374 mutant endopin 2 cDNAs in the pET19b vector. The C232A and C374A constructs were each digested with *Alw*NI. Ligation of the resultant 4.6 kb cDNA fragment from C232A that contains the Ala substitution for Cys-232, and the resultant 2.2 kb cDNA fragment from C372A that contains the Ala substitution for Cys-374, generated the double-Cys mutant known as C232A/C374A.

Expression of wild-type and mutant forms of endopin 2 in *E. coli* was performed as described previously (*4*, *12*). Recombinant proteins were purified by affinity chromatography on a Ni^{2+} column, resulting in purified forms of endopin 2 that were analyzed on SDS−PAGE gels [as described previously (*4*, *12*)].

Inhibition of Elastase by Wild-Type and Mutant Forms of Endopin 2. Inhibition of elastase by endopin 2 (wild-type or mutant forms) was assayed in the absence or presence of the reducing agent dithiothreitol (1 mM). Recombinant endopin 2, with an N-His tag, was expressed in *E. coli* and purified as described previously (*4*). To assess endopin 2 inhibition of elastase, endopin 2 was preincubated with porcine elastase (20 ng/100 μL assay, Worthington, Freehold, NJ) in 50 mM Tris-HCl, pH 8.0, 0.005% Triton X-100, and 1 mM DTT for 15 min at room temperature. The peptide-MCA substrate Suc-Ala-Ala-Ala-MCA was added to a final concentration of 100 μM, and the reaction was incubated at 24 °C for 30 min. Elastase activity was monitored by the production of fluorescent AMC (aminomethylcoumarin-amide) as described previously (*4*). It is noted that 1 mM DTT has a minimal effect on elastase activity, since 80% elastase activity was observed in the presence of 1 mM DTT compared to assay of elastase in the absence of DTT (100% activity). Endopin 2 (wild-type) inhibition of chymotrypsin (*8*), trypsin (*8*), plasmin (*13*), thrombin (*14*), and furin (*15*) was also tested, with assay of these proteases as described previously (*4*) with the respective substrates Suc-Ala-Ala-Pro-Phe-MCA, Z-Phe-Arg-MCA, Boc-Glu-Lys-Lys-MCA, Boc-Val-Pro-Arg-MCA, and pGlu-Arg-Thr-Arg-Arg-MCA.

After incubation of endopin 2 with elastase and measurement of Suc-Ala-Ala-Ala-MCA cleaving activity, the identical reaction samples were analyzed for SDS-stable complexes of endopin 2 and elastase. At the end of the incubation period, PMSF (phenylmethanesulfonyl fluoride) was added to a final concentration of 5 mM to inactivate elastase activity, and 5 μL of the sample was subjected to nonreducing SDS−PAGE gels (without reducing agent and without heating of samples) and western blotting with anti-endopin 2 serum using the ECL Plus enhanced chemiluminescence detection system (Amersham Life Sciences, Piscataway, NJ), as described previously (*4*, *16*, *17*).

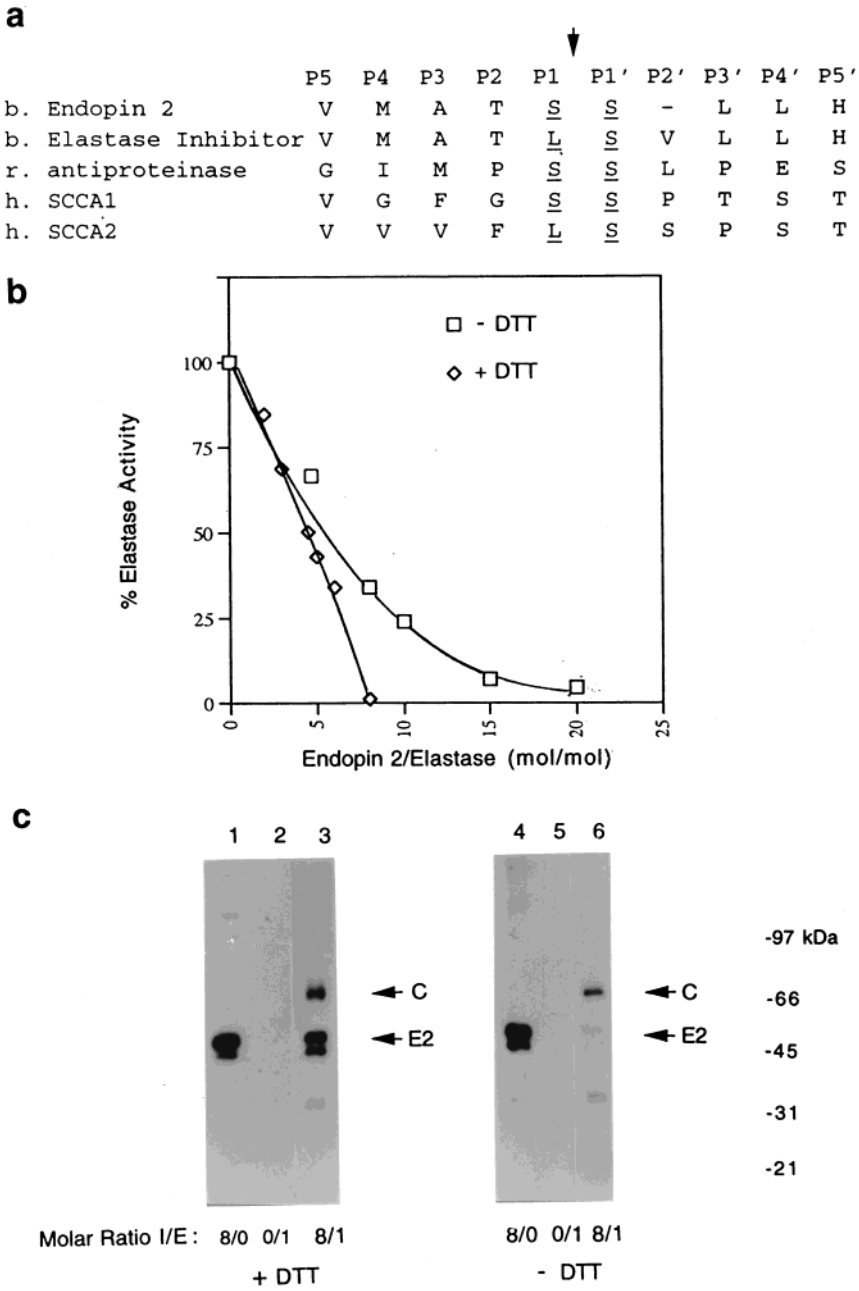

FIGURE 1: DTT dependence of endopin 2 inhibition of elastase. (a) RSL domain of endopin 2. Alignment of the RSL domain of endopin 2 with bovine elastase inhibitor, rabbit antiproteinase inhibitor, and human SCCA1 and SCCA2 serpins is illustrated. (b) Stoichiometry of endopin 2 inhibition of elastase under nonreducing and reducing conditions. Endopin 2 inhibition of elastase was tested at different molar ratios of inhibitor/enzyme in the presence (◇) or absence (□) of DTT (1 mM). Control elastase activity was measured with or without DTT (1 mM) for the plus and minus DTT curves, respectively. (c) SDS-stable complexes of endopin 2 and elastase. Endopin 2 complexes with elastase were assessed by western blots with anti-endopin 2 serum. The formation of SDS-stable complexes of endopin 2 and elastase was evaluated at a molar ratio of inhibitor/enzyme of 8/1 (lanes 3, and 6), in the presence (lanes 1−3) or absence (lanes 4−6) of DTT (1 mM). Endopin 2 (E2) alone (lanes 1 and 4) and complexes (C) with elastase are indicated by arrows.

Bis(maleimidoethane) Chemical Modification of Endopin 2. BMOE [bis(maleimido)ethane; Pierce, Rockford, IL] was used for chemical modification of sulfhydryl groups of Cys residues within endopin 2. Purified wild-type and mutant forms of endopin 2 (100 μg each) were each incubated in 10 mM Tris-HCl, pH 7.2, and 0.1 mM DTT (total volume of 198 μL) for 10 min at 4 °C. The sample was then divided into two aliquots of 99 μL. One microliter of 50 mg/mL BMOE (in dimethyl sulfoxide, DMSO) was added to one 99 μL aliquot, and 1 μL of DMSO was added to the other aliquot as a control. These samples were incubated at 4 °C for 3.5 h and then at room temperature for 1.5 h. Samples were dialyzed against 2 × 500 mL of 50 mM Tris-HCl, pH 7.5, overnight at 4° C. Modified endopin 2 was subjected to SDS−PAGE (12% polyacrylamide), as described previously (*4*, *12*).

RESULTS

DTT Enhances Endopin 2 Inhibition of Elastase. Homology of the reactive site loop (RSL) of endopin 2 with bovine elastase inhibitor (*6*) and rabbit α_1-antiproteinase inhibitor (*7*) (Figure 1a) suggested that endopin 2 should inhibit elastase. Therefore, endopin 2 was expressed in *E. coli* to

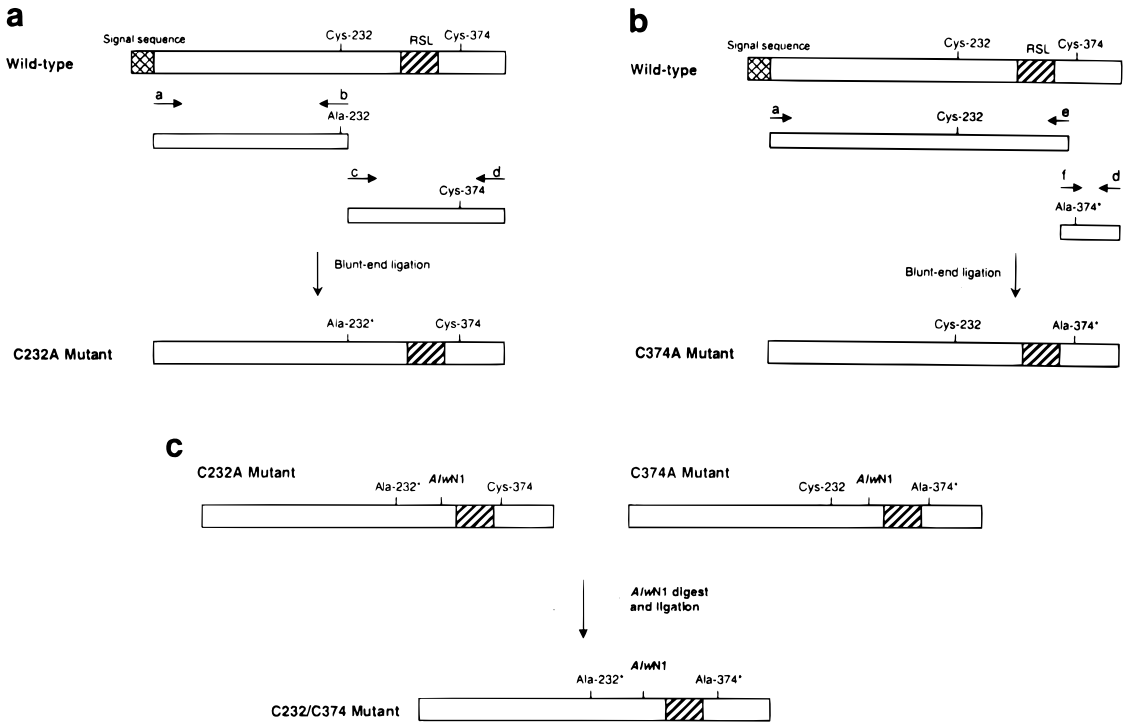

FIGURE 2: PCR mutagenesis to generate endopin 2 mutant constructs. (a) C232A single-cysteine mutant. Substitution of Ala for Cys-232 to generate the C232A mutant form of endopin 2 was achieved by PCR with primers a and b, which incorporated the mutation, and by PCR of the endopin 2 cDNA and with primers c and d. Ligation of resultant PCR DNA products generated the C232A mutant construct in the pET19 expression vector. (b) C374A single-cysteine mutant. Substitution of Ala for Cys-374 to generate the C374A mutant was achieved by PCR of the endopin 2 cDNA with primers a and e, and by PCR with primers f and d with incorporation of the mutation by primer f. Ligation of resultant PCR products generated the C374A mutant in the pET19b vector. (c) C232A and C374 double-cysteine mutant. The double mutant with substitution of both Cys residues by Ala was obtained by *Alw*NI digests of the C232A and C374A single mutants, followed by ligation at the *Alw*NI site.

assess endopin 2 inhibition of elastase. Recombinant endopin 2 was expressed as an NH_2-His-tagged form and was affinity purified by a Ni^{2+} column. Endopin 2 inhibition of elastase was assessed in the presence or absence of the reducing agent DTT (dithiothreitol). DTT enhanced endopin 2 inhibition of elastase, assessed by determining the stoichiometry of inhibition, that is, the molar ratio of inhibitor to protease for complete inhibition. In the presence of DTT, complete inhibition was observed at a molar ratio of endopin 2 to elastase of 8/1 (Figure 1b). In contrast, in the absence of reducing agent, complete endopin 2 inhibition of elastase required a higher molar ratio of 25/1. Effective inhibition in the presence of DTT was indicated by the low level of endopin 2, 32 nM, required to reduce elastase activity by 50%. These data demonstrate that endopin 2 inhibits elastase in a DTT-dependent manner.

Formation of SDS-stable complexes of a serpin and its target protease is a characteristic property of serpins (*1*, *2*). Experiments demonstrated that formation of endopin/elastase complexes was enhanced in the presence of DTT (Figure 1c). Complex formation was demonstrated by a prominent band of retarded electrophoretic mobility representing endopin 2/elastase complexes (at a molar ratio I/E = 8/1), detected on nonreducing SDS−PAGE gels subjected to anti-endopin 2 western blots. In the absence of DTT, lower levels of endopin 2/elastase complexes were detected (at the same molar ratio of I/E = 8/1), and endopin 2 appeared to be less stable since it was partially degraded. It was noted that in the presence of DTT, western blots detected endopin 2 as a doublet consisting of a major band of 49 kDa and a lesser band of 45 kDa; however, nearly equivalent levels of the doublet bands were detected in the absence of DTT. These data suggested that DTT-induced reduction promotes a change in endopin protein conformation to retard its electrophoretic mobility.

In contrast to effective inhibition of elastase, endopin 2 did not inhibit chymotrypsin (at a molar ratio of inhibitor/enzyme of 10/1), although endopin 2 possesses 60% homology with the α_1-antichymotrypsin that inhibits chymotrypsin. Endopin 2 moderately inhibited (50% inhibition) subtilisin (I/E = 10/1); in addition, endopin 2 did not inhibit basic residue-cleaving serine proteases trypsin, plasmin, thrombin, or furin (data not shown). These findings demonstrate the preference of endopin 2 to inhibit elastase, rather than other proteases.

Expression of Wild-Type and Mutant Forms of Endopin 2. Wild-type endopin 2 (without signal sequence) contains two cysteine residues at amino acid positions 232 and 374. To determine whether one or both of these cysteine residues may be responsible for the DTT sensitivity of endopin 2, mutant forms of endopin 2 lacking one or both of the cysteine residues were generated by site-directed mutagenesis and expression in *E. coli* (Figure 2). Cysteine at position 232 was converted to alanine to generate the C232A mutant. Similarly, cysteine at position 374 was substituted with alanine to generate the C374A mutant. In addition, both cysteines were substituted with alanine to generate the C232A/C274A double mutant.

Wild-type and mutant forms of endopin 2 were expressed in *E. coli* as NH_2-His-tagged fusion proteins. Expression was

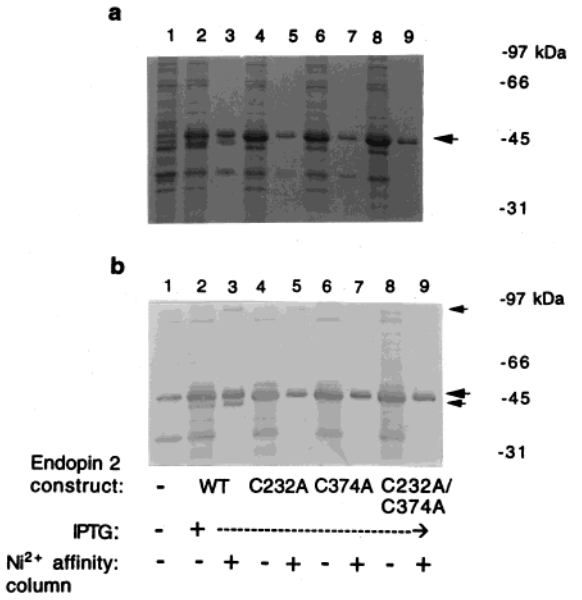

FIGURE 3: Expression and purification of mutant forms of endopin 2. Expression of wild-type and mutant forms of endopin 2 in *E. coli* was induced with IPTG. Purification of N-His-tagged endopin 2 forms was achieved by affinity chromatography on a Ni^{2+} column. Recombinant endopin 2 forms were analyzed by detection of protein by Coomassie blue-stained SDS−PAGE gels (panel a) and by western blots with anti-endopin 2 serum (panel b). The legend for lanes 1−9 is indicated below the figure.

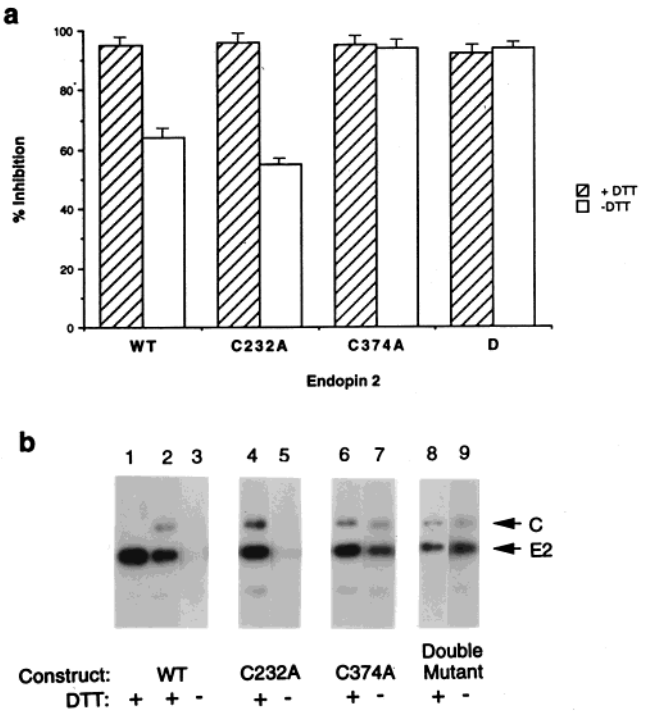

FIGURE 4: Cys-374 confers DTT sensitivity of endopin 2 inhibitory activity. (a) Inhibition of elastase by wild-type and mutant forms of endopin 2. Inhibition of elastase in the presence and absence of DTT (1 mM) by wild-type and mutant forms of endopin 2 was tested at inhibitor/enzyme molar ratios of 10/1. Values were determined in triplicate that varied by less than 5−10% (bar graph shows mean ± SEM). (b) Formation of SDS-stable complexes of elastase with variant forms of endopin 2. The formation of SDS-stable complexes of wild-type and mutant forms of endopin 2 with elastase (at I/E ratio of 10/1) was analyzed in the presence and absence of DTT (1 mM) by nonreducing SDS−PAGE gels and western blots with anti-endopin 2 serum.

induced with IPTG, demonstrated by increased production of wild-type and mutant forms of endopin 2 of approximately 49 kDa on SDS−PAGE gels (nonreducing gels, stained with Coomassie blue) (Figure 3a). Affinity chromatography on Ni^{2+} columns resulted in purified wild-type endopin 2, C232A mutant, C374A mutant, and the C232A/C374A double mutant (Figure 3a). These wild-type and mutant forms of endopin 2 were recognized in western blots by the anti-endopin 2 serum (Figure 3b). High levels (milligram quantities) of purified recombinant N-His-tagged wild-type and mutant forms of endopin 2 were obtained.

Western blots on nonreducing SDS−PAGE gels indicated purified wild-type endopin 2 as a primary 49 kDa band and as a minor 45 kDa band (Figure 3b). Moreover, reduction of the wild-type endopin 2 resulted in a single 49 kDa form (lane 1 of Figure 4b, lane 1 of Figure 5c). If the 45 kDa band possesses disulfide bonds between Cys-232 and Cys-374, then disruption of disulfide bonds by mutagensis of either Cys residue should result in only the 49 kDa form. Indeed, mutagenesis of one or both Cys residues to Ala resulted in detection of only the 49 kDa form of endopin 2 (Figure 3). These data are consistent with the presence of an intramolecular disulfide bond between Cys-232 and Cys-374 for the 45 kDa form of wild-type endopin 2, and absence of disulfide bonds for the 49 kDa form. These data suggest that the two cysteine residues of endopin 2 may form disulfide bonds that modify the electrophoretic mobility and conformation of endopin 2.

Western blots of endopin 2 on nonreducing SDS−PAGE gels also indicated a minor 95 kDa band that may represent dimers of endopin 2 (Figure 3b). The 95 kDa band was present in wild-type and C232A forms of endopin 2 but not in the C374A or double mutant forms. These results would be consistent with the presence of an intermolecular disulfide bond between Cys-374 from two molecules of endopin 2 that could generate a dimer.

Cys-374 Confers DTT Sensitivity of Endopin 2 Inhibitory Activity. Wild-type and mutant forms of endopin 2 were tested for DTT-dependent inhibition of elastase at a I/E molar ratio of 10/1 (Figure 4a). Wild-type and C232A forms of endopin 2 showed enhanced inhibitory activity in the presence of DTT. However, the C374A mutant that lacks Cys-374 was not sensitive to DTT. Consistent with these results, the double C232A/C374A mutant was not sensitive to DTT. Furthermore, DTT promoted formation of SDS-stable complexes of wild-type or C232A forms of endopin 2 with elastase (Figure 4b). Without DTT, these wild-type and C232A forms were apparently degraded by elastase, since these forms of endopin 2 were not detected on the SDS−PAGE gels after incubation with elastase. Moreover, since the inhibitory activities of mutants lacking Cys-374 were not sensitive to DTT (C374A and C232A/C374A mutants, Figure 4a), they were predicted to show similar levels of SDS-stable complexes with elastase in the presence or absence of DTT, as illustrated in Figure 4b. These results suggest that the oxidation state of Cys-374 acts as a switch that determines whether endopin 2 functions as a serpin inhibitor or substrate. These results demonstrate that Cys-374 is responsible for the DTT-sensitive inhibitory activity of endopin 2.

BMOE Chemical Modification of Endopin 2 Modifies Inhibitory Activity. Chemical modification of Cys-374 by

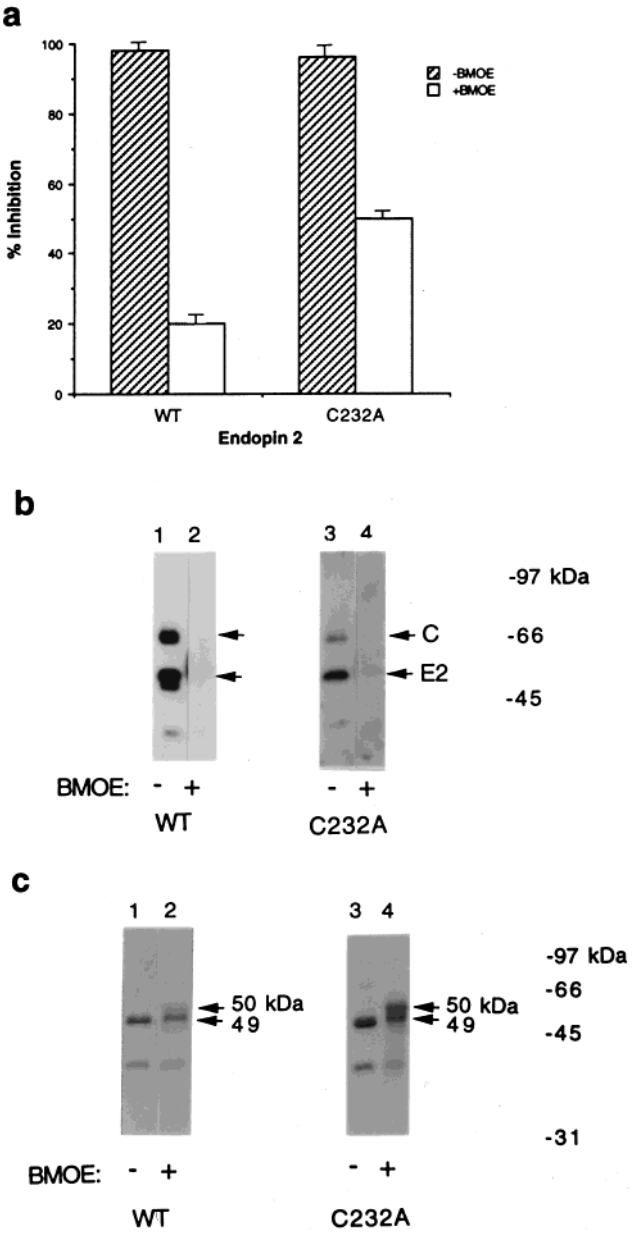

FIGURE 5: BMOE modification alters DTT sensitivity of endopin 2 inhibitory activity. (a) Effect of BMOE treatment on inhibitory activity of wild-type and mutant forms of endopin 2. Inhibition of elastase (in the presence of DTT) by BMOE-treated wild-type and C232A mutant forms of endopin 2 was examined. Percent inhibition was determined in triplicate, shown as the mean ± SEM. (b) Endopin 2 complex formation with elastase. The effect of BMOE treatment (lanes 2 and 4) on the ability of wild-type (lanes 1 and 2) or C232A (lanes 3 and 4) forms of endopin 2 to form SDS-stable complexes with elastase was assessed on nonreducing SDS−PAGE gels subjected to anti-endopin 2 western blots. (c) BMOE alters electrophoretic mobility of endopin 2. Wild-type (lanes 1 and 2) and C232A (lanes 3 and 4) forms of endopin 2 that were incubated without (lanes 1 and 3) or with BMOE (lanes 2 and 4) were analyzed on reducing SDS−PAGE gels (100 mM β-ME in sample preparation buffer) with respect to relative electrophoretic mobilities.

BMOE was utilized to further assess its role for the inhibitory activity of endopin 2 (Figure 5a). BMOE [bis(maleimido)-ethane] is a sulfhydryl-reactive chemical reagent that conjugates thiol-containing molecules. BMOE reduced the ability of endopin 2 (wild-type) to inhibit elastase by 80%, with inhibitor/enzyme at a molar ratio of 10/1 and assays conducted in the presence of DTT. BMOE treatment of the C232A mutant also reduced the ability of endopin 2 to inhibit elastase by approximately 50%, suggesting the importance of Cys-374 for optimum endopin 2 activity. In contrast, BMOE treatment of the C374A mutant had no effect on its inhibitory activity (data not shown), indicating that Cys-232 did not alter endopin 2 activity. Moreover, BMOE prevented wild-type and C232A forms of endopin 2 from forming SDS-stable complexes with elastase (Figure 5b). These results provide further support for the role of Cys-374 in mediating the DTT sensitivity of endopin 2.

BMOE modified the electrophoretic mobility of wild-type and the C232A mutant form of endopin 2. Wild-type endopin 2 was observed as a single 49 kDa band in the absence of BMOE (in reducing SDS−PAGE gels; Figure 5c, lane 1). However, after incubation with BMOE, endopin 2 was observed as a new 50 kDa form, as well as the 49 kDa form (lane 2). Similarly, BMOE treatment of the C232A mutant also resulted in the new 50 kDa band (Figure 5c, lane 4). However, incubation of the C374A mutant with BMOE, which presumably involves modification of Cys-232, had no effect (data not shown). These results suggest a role for Cys-374 in modifying the electrophoretic mobility and conformation of endopin 2.

Glutathione as an Endogenous Regulator of Endopin 2. The ability of glutathione, an endogenous reducing agent in biological tissues (*18*−*20*), was tested as an activator of endopin 2. Glutathione (2 mM) was as effective as DTT (1 mM) for optimizing the inhibitory activity of endopin 2 (Figure 6a). Stimulation of endopin 2 with millimolar concentrations of glutathione (2 mM) is consistent with endogenous millimolar levels of glutathione in various cell types and tissues (*18*−*20*). As expected, reduced glutathione enhanced the formation of endopin 2 complexes with elastase (Figure 6b). Moreover, similar to DTT, glutathione converted the doublet of 49 and 45 kDa bands of wild-type endopin 2 to a single 49 kDa band (Figure 6c). These results demonstrate that glutathione concomitantly modifies the electrophoretic mobility of endopin 2 and enhances the inhibitory activity of endopin 2. These results demonstrate that glutathione could modify endopin 2 activity in vivo.

DISCUSSION

Primary sequence analysis of the serpin endopin 2 predicts that it would inhibit elastase, based on homology of the reactive site loop of endopin 2 to bovine elastase inhibitor and α_1-antiproteinase serpins that inhibit elastase (*6*, *7*). In addition, two Cys residues within endopin 2 may potentially influence the inhibitory activity of endopin 2, since the state of oxidation and reduction of cysteine residues can vary. To test these predicted properties of endopin 2, expression and mutagenesis, combined with chemical modification, were utilized to demonstrate that Cys-374 is required for DTT-dependent inhibition of elastase. Inhibition of elastase by endopin 2 was enhanced in the presence of DTT, demonstrated by assessing the stoichiometry of inhibition. In the presence of DTT, complete inhibition of elastase occurred at a molar ratio of inhibitor/enzyme of 8/1; however, less effective inhibition occurred in the absence of DTT. Mutagenesis of endopin 2 indicated that Cys-374, but not Cys-232, was responsible for the DTT sensitivity of endopin 2. Chemical modification of sulfhydryl groups by BMOE provided further support for the importance of Cys-374 for

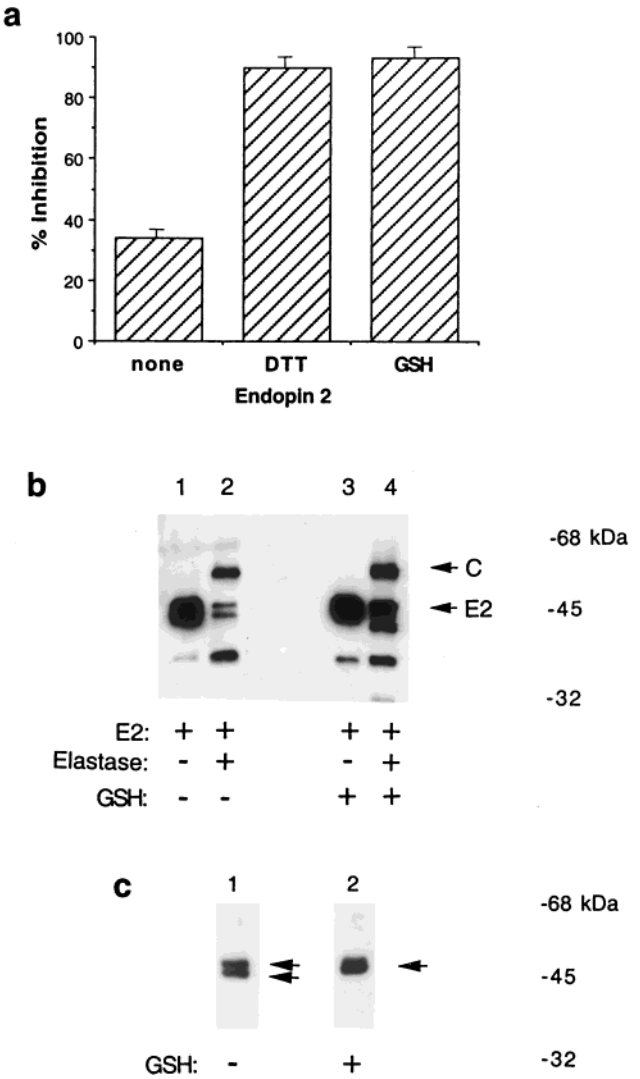

FIGURE 6: Glutathione stimulates endopin 2 inhibitory activity. (a) GSH increases endopin 2 inhibition of elastase. Endopin 2 (wild-type) inhibition of elastase activity was examined without DTT, with DTT (1 mM), or with reduced glutathione (2 mM). Assays were conducted in duplicate, shown as mean ± SEM. (b) GSH enhances formation of endopin 2 and elastase complexes. Endopin 2 (E2) and elastase were incubated at a molar ratio of 10/1 in the absence or presence of GSH (2 mM), and formation of SDS-stable endopin/elastase complexes (C) was observed by gel electrophoresis. (c) Modified electrophoretic mobility of endopin 2 in the presence of GSH. Wild-type endopin 2 was incubated without (lane 1) or with (lane 2) GSH (2 mM), as described under Materials and Methods, and endopin 2 was subjected to reducing SDS−PAGE.

endopin 2 activity. In addition, effective inhibition was accompanied by the formation of prominent SDS-stable complexes of endopin 2 and elastase, a characteristic property of serpins (*1*, *2*). Alterations in electrophoretic mobility of mutant forms of endopin 2, and BMOE modification of the C232A mutant, suggested that Cys-374 influences the conformation and activity of endopin 2. Moreover, glutathione, present within biological tissues, serves as an effective reducing agent for optimum endopin 2 activity, suggesting that endopin 2 in vivo could be modified by glutathione. These findings demonstrate the importance of Cys-374 for the DTT-dependent endopin 2 inhibition of elastase.

High homology of the RSL domain of endopin 2 with bovine elastase inhibitor (*6*) compared to other serpins (*7*−*10*) suggested that endopin 2 may selectively inhibit elastase, compared to other serine proteases. Tests of protease inhibition by endopin 2 showed that it did not inhibit α_1-antichymotrypsin (ACT) that inhibits chymotrypsin (*8*), although endopin 2 possesses homology (60%) with ACT. In addition, endopin did not inhibit the serine proteases trypsin, plasmin, thrombin, or furin. Thus, endopin 2 possesses selectivity for inhibiting elastase.

The primary sequence of mature endopin 2 (without signal sequence) indicates two Cys residues at amino acid positions 232 and 374 (*3*). To determine whether these Cys residues participate in the DTT sensitivity of endopin 2, mutant forms of endopin 2 with substitution of one or both Cys residues with Ala were generated by site-directed mutagenesis. Wild-type and mutant forms of recombinant endopin 2 were expressed as NH_2-His-tagged proteins in *E. coli* and were purified by affinity purification on Ni^{2+} columns. The wild-type and C232A mutant forms both displayed DTT-sensitive inhibition of elastase (at I/E = 10). Substitution of Cys-374 with Ala resulted in loss of DTT sensitivity. These results suggested that the Cys-374 present within the C232A mutant was responsible for the DTT sensitivity. These results indicated that Cys-374 is necessary for DTT-sensitive inhibition of elastase by endopin 2.

Further evidence for involvement of Cys-374 in the DTT-sensitive activity of endopin 2 was demonstrated by chemical modification of sulfhydryl groups by BMOE [bis(maleimido)ethane]. Incubation of wild-type or the C232A mutant forms of endopin 2 with BMOE reduced its inhibitory activity (in the presence of DTT), suggesting the importance of sulfhydryl groups for activity. However, incubation of the C374A mutant with BMOE, which presumably involves modification of Cys-232, had no effect. Thus, chemical modification by BMOE provides further evidence for the importance of Cys-374 for the inhibitory activity of endopin 2.

Endopin 2 displayed altered electrophoretic mobilities when Cys-374 was modified by mutagenesis or chemical modification, suggesting that Cys-374 influences the conformation of endopin 2. Purified wild-type endopin 2 was observed as major and minor bands of 49 and 45 kDa, respectively, on nonreducing SDS−PAGE gels. However, the mutant forms of endopin 2 that lack one or both Cys residues were detected only as the 49 kDa band. Also, under reducing conditions, wild-type endopin 2 was observed only as a 49 kDa band. These data are consistent with the presence of a disulfide bond between Cys-232 and Cys-374 for the 45 kDa form of endopin 2. Disruption of the disulfide bond by mutagenesis of Cys residues, or by reduction of the wild-type endopin, results in the 49 kDa form. Significantly, reduction of the predicted disulfide bond within endopin 2 is necessary for obtaining only reduced Cys-374, not reduced Cys-232, for the DTT-sensitive inhibitory activity of endopin 2. Significantly, the reduced Cys-374 is the critical structural feature that is required for optimum endopin 2 activity.

It is noted that endopin 2 may appear as a doublet with different proportions of upper and lower 49 and 45 kDa bands, in the presence of reducing conditions by 1 mM DTT. Specifically, in the absence of DTT (Figure 1c, lane 4), similar levels of each band of the doublet were observed. However, in the presence of 1 mM DTT (Figure 1c, lane 1), a higher proportion of the upper 49 kDa band, compared to the lower 45 kDa band, was detected. These results, combined with the different electrophoretic mobilities of

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                                  +1
h.AT      MPSSVSWGIL -LLAGLCCLV PVSLAEDPQG DAAQKTDTSH HDQDHPTFNK ITPNLAEFAF  59
h.ACT     MERMLPILAL GLLAAGFCPA VLCHPNSPLD EENLTQENQD RGTHVDLGLA SA-NVD-FAF
b.Endo.2  -FPERTSFLL ALGLLVSGFC SRVHCLPENV TPEEQYKGTS VDGHS---LA SSNTD--FAF
                                  +1
                        s6B
h.AT      SLYRQLAHQS NSTNIFFSPV SIATAFAMLS LGTKADTHDE ILEGLNFNLT EIPEAQIHEG 119
h.ACT     SLYKQLVLKA PDKNVIFSPL SISTALAFLS LGAHNTTLTE ILKGLKFNLT ETSEAEIHQS
b.Endo.2  SLYKQLALKD PNKNVIFSPL SISIALGFLS LGGHDHTVTE ILEGLKFNLT ETPETEIHQG

                        <   s2A      >                   < s1A  >
h.AT      FQELLRTLNQ PDSQLQLTTG NGLFLSEGLK LVDKFLEDVK KLYHSEAFTV NFGDTEEAKK 179
h.ACT     FQHLLRTLNQ SSDELQLSMG NAMFVKEQLS LLDRFTEDAK RLYGSEAFAT DFQDSAAAKK
b.Endo.2  FQHLLQTFNQ PSNQLQLSVG NAMFVSEELK LLDKFRKDAE AFYASEVLST NFKDSEAAVK

                                    <     s3A     >          <   s4C
h.AT      QINDYVEKGT QGKIVDLVKE LDRDTVFALV NYIFFKGKWE RPFEVKDTEE EDFHVDQVTT 239
h.ACT     LINDYVKNGT RGKITDLIKD LDSQTMMVLV NYIFFKAKWE MPFDPQDTHQ SRFYLSKKKW
b.Endo.2  LINEYVKNKT HGKIEKLFND LSVLTNLILL NYIFFKAQWK TPFNPNHTYE SEFHVSQNER

          ><     s3C       >     <  s2B    > <  s3B >
h.AT      VKVPMMKRLG MFNIQHCKKL SSWVLLM-KY LGNATAIFFL PDEGKLQHLE NELTHDIITK 298
h.ACT     VMVPMMSLHH LTIPYFRDEE LSCTVVELKY TGNASALFIL PDQDKMEEVE AMLLPETLKR
b.Endo.2  VIVPMMTLYL ETPYFR-DEE LGCTLVEVTF TRNDRGLFIL PDEGKMQDLE AKLTPETLTR

                 <   s2C    >< s6A >                          < s5A
h.AT      FLENEDRRSA S-LHLPKLSI TGTYDLKSVL GQLGITKVFS NGADLSGVTE EAPLKLSKAV 357
h.ACT     WRDSLEFREI GELYLPKFSI SRDYNLNDIL LQLGIEEAFT SKADLSGITG ARNLAVSQVV
b.Endo.2  WRNSLQPRLI HRLRLSRFSI SSHYQLKDIL SQLGIKKIFT SDADFSGITD DHKLADSHVI

               ><     s4A          >*   < s1C > <   s4B   >    <    s5B   >
h.AT      HKAVLTIDEK GTEAAGAMFL EAIPMSIPPE VKFNKPFV-- FLMIEQNTKS PLFMGKV--V 413
h.ACT     HKAVLDVFEE GTEASAATAV KITLLSALVE TRTIVRFNRP FLMIIVPTDT QNIFFMSKVT
b.Endo.2  HKPVLDVGEE GTEGAAVTAV VMATSSLLHT LTVSFNRPFL LSIFCKETQS IIFVGKVTNP

h.AT      NPTQK                                                          418
h.ACT     NPKQA
b.Endo.2  KEA
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FIGURE 7: Alignment of endopin 2 primary sequence with α_1-antitrypsin and α_1-antichymotrypsin. Alignment of the primary sequence of endopin 2 with human α_1-antitrypsin (AT) and human α_1-antichymotrypsin (ACT) is illustrated, with indication of the β-sheet domains (within the brackets < >) present within human AT. The +1 residue above the AT sequence indicates the NH_2-terminal residue of mature AT without its signal sequence. The +1 residue of mature endopin 2 begins at LPENV..., indicated (by +1) below the bovine endopin 2 sequence (b.Endo.2). The asterisk indicates the P1 residue within the reactive site loop (RSL) of each serpin that may be recognized by respective target proteases.

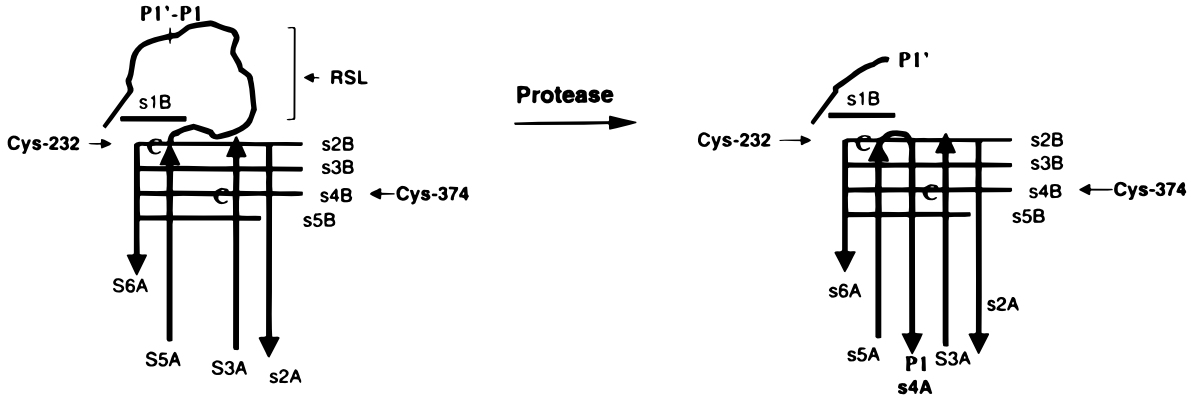

FIGURE 8: Cys-374 located near s4A strand insertion within β-sheet A of a prototypic serpin. The relative locations of Cys-374 and Cys-232 are illustrated, based on homology of endopin 2 with known β-sheet domains of α_1-antitrypsin and α_1-antichymotrypsin (*11*, *21*, *22*). The position of Cys-374 is predicted to be located on the s4B sheet, where the cleaved s4A strand becomes inserted between the s3A and s5A β-sheets. Reduced Cys-374 has been demonstrated to enhance the inhibitory activity of endopin 2. It is proposed that modification of Cys-374 by disulfide bond formation with Cys-232, substitution with Ala, or chemical modification by BMOE may interfere with s4A strand insertion to reduce protease inhibition by endopin 2. Effective s4A strand insertion is involved in the inhibitory mechanism of serpins (*11*, *21*, *22*).

mutant endopin 2 (Figure 3), demonstrate that 1 mM DTT promotes the reduction of disulfide bonds between Cys-232 and Cys-374 to generate reduced endopin 2 that is observed as a 49 kDa band of retarded electrophoretic mobility.

Further evidence for participation of Cys-374 in the conformation of endopin 2 was demonstrated by BMOE

modification of wild-type and C232A mutant forms of endopin 2. BMOE altered the electrophoretic mobility (on nonreducing SDS−PAGE) of wild type and C232A, which both contain Cys-374. However, BMOE did not alter the electrophoretic mobility of the C374A mutant, which lacks Cys-374. These data provide additional evidence for a role of Cys-374 in the conformational status of endopin 2.

Recent studies show that endopin 2 in vivo is localized to glutathione-containing neurosecretory vesicles of adrenal medullary chromaffin cells (*4*). Glutathione (GSH) is known to be an endogenous reducing agent (*18*−*20*). GSH, indeed, effectively enhanced the inhibitory activity of endopin 2 at millimolar concentrations, which represents in vivo levels of glutathione within secretory vesicles (*18*−*20*). Similar to DTT, GSH modifies the electrophoretic mobility and conformation of endopin 2, which presumably participates in GSH-induced increases in endopin/elastase complexes. Therefore, maximal endopin 2 activity in vivo can be achieved by the presence of endogenous glutathione.

Endopin 2 possesses high homology (55%) with human liver α_1-antichymotrypsin (ACT) and moderate homology with human α_1-antitrypsin (AT) (*3*). The determined structures (by X-ray crystallography) of AT and ACT serpins (*11*, *21*, *22*) provide prototypic models for structural features of serpins. Alignment of the primary sequence of endopin 2 with the serpins AT and ACT (*11*, *21*, *22*) (Figure 7) predicts that Cys-374 (P20′ residue of the reactive site loop) of endopin 2 may reside within the s4B β-sheet domain and that Cys-232 resides in the s2B sheet of a prototypic serpin represented by AT. It is known that, upon cleavage of the RSL domain of a serpin, the resultant s4A strand becomes inserted between the s3A and s5A β-sheets (Figure 8). Indeed, preliminary results demonstrate cleavage of endopin 2 within its RSL domain by elastase. On the basis of prototypic models of serpins, Cys-374 at the s4B sheet may be situated in the vicinity of s4A strand insertion (*11*, *22*, *23*) (Figure 8). Because s4A strand insertion is involved in the inhibitory mechanisms of serpins (*11*, *21*, *22*), Cys-374 may influence s4a strand insertion and modify the inhibitory activity of endopin 2. Clearly, reduced Cys-374 participates in the DTT sensitivity of endopin 2 inhibitory activity.

Moreover, modification of Cys-374 by disulfide bond formation with Cys-232, mutagenesis, or chemical modification by BMOE alters the electrophoretic mobility and conformation of endopin 2 in a manner that may influence s4A strand insertion. Interestingly, the sheep α_1-proteinase inhibitor and bovine α_1-antrypsin both possess a cysteine residue at position 374 (*24*, *25*). It will be interesting to assess through mutagenesis studies whether Cys-374 has similar roles for influencing inhibitory activities of these related serpins.

Overall, results from this study demonstrate that the inhibitory activity of endopin 2 against elastase was enhanced with DTT or the reducing agent glutathione that is present in biological tissues (*18*−*20*). Site-directed mutagenesis and chemical modification of cysteine residues demonstrated that Cys-374 is the critical residue responsible for the DTT-sensitive nature of endopin 2. Changes in the conformation of endopin 2 upon mutagenesis or chemical modification of Cys-374 (demonstrated by modified electrophoretic mobilities of endopin 2) suggested that Cys-374 influences the structure and activity of endopin 2. Moreover, Cys-374 in reduced form enhances endopin 2 inhibitory activity. The increased protease inhibitory activity of endopin 2 under reducing conditions suggests that it could be influenced by reducing environments in vivo in certain cells and tissues (*18*−*20*).

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