An Inhibitor of Nuclear Scaffold Protease Blocks Chemical Transformation of Fibroblasts

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Abstract

A nuclear scaffold (NS) protease has previously been implicated in production of the M, 46,000 ATP-binding protein in NS (which may acquire nucleoside triphosphatase activity and participate in nucleocytoplasmic transport) by cleavage of a subset of lamins A/C. In a preceding paper (G. Clawson, L. Norbeck, C. Hatem, C. Rhodes, P. Amiri, J. McKerrow, S. Patierno, and G. Fiskum, Cell Growth & Differ., 3: 827–838), this NS protease was identified as a novel, Ca2+-regulated serine protease, which was found only in the NS and which appears to represent a unique multicatalytic protease complex. Based upon its predominantly chymotrypsin-like substrate preference, a peptide-chloromethylketone inhibitor (succinyl-AAPF-chloromethylketone, AAPFcmk) was identified. AAPFcmk showed a $K_i = 56$ nm for the NS protease versus 1.4 $\mu$m for the endoplasmic reticulum (ER) catalytic activity. Treatment of C3H/10T1/2 mouse embryo fibroblasts with 1 $\mu$m AAPFcmk produced effects which were confined to the nuclear (and to a lesser extent the endoplasmic reticulum) compartment. In this report, we examine the effects of the AAPFcmk inhibitor on cellular transformation and growth. Growth of C3H/10T1/2 cells was decreased by 34% and 56% at 25 $\mu$m and 5 $\mu$m AAPFcmk, respectively. Growth inhibition occurred without any major change in DNA content distribution, suggesting effects throughout the cell cycle. Growth inhibition was not observed at lower ($\leq 10$ $\mu$m) concentrations, which decreased transformation of C3H/10T1/2 fibroblasts in a dose-dependent manner by up to 90%, even at femtomolar concentrations of AAPFcmk (in the absence of growth inhibition). Inclusion of irrelevant inhibitors was without affect. These results suggest that the NS protease may play an important role in acquisition of the transformed phenotype.

Introduction

Recent investigations have documented a protease activity that is associated with the NS$^2$ (1–5). This protease produces a M, 46,000 ATP-binding protein from the NH$_2$ terminus of lamins A/C by cleavage at Y$_{376}$ (3, 6). This lamin fragment appears to represent an authentic in vivo component (6, 7) and may represent the nucleoside triphosphatase (8, 9) thought to participate in nucleocytoplasmic transport (10–18). In a preceding paper (1), we identified the NS protease as a novel Ca$^{2+}$-regulated serine protease which was present only in the NS (in fibroblasts and hepatocytes) and which appears to represent a unique MPC. Based upon substrate utilization, a potent chloromethylketone inhibitor (succinyl-AAPF-chloromethylketone, AAPFcmk) was identified. The effects of 1 $\mu$m AAPFcmk were confined to the NS ($K_i = 56$ nm), with a lesser, later effect on chymotryptic-like activity in the ER fraction ($K_i = 1.4$ $\mu$m). Since previous investigations have shown that transformation by chemical carcinogens is associated with early, permanent alterations in nuclear size and ploidy (see Ref. 19), nucleocytoplasmic RNA transport (20–25), and NS nucleoside triphosphatase (20, 21), all of which may involve NS protease activity, we examined the potential importance of the NS protease in cell transformation.

Results and Discussion

Based on previous results (1) with NS protease from rat liver, a limited spectrum of benzyl substrates were tested for hydrolysis by the NS protease from C3H/10T1/2 mouse embryo fibroblasts. Substrates with F in P, particularly AAPFsb, were again found to be the most effectively hydrolyzed substrates tested, and substrate parameters and preferences were similar, although the trypsin-like activity was increased with the fibroblast NS protease (for instance, VLKsb hydrolysis was 55% of that with AAPFsb), whereas ZYsb was less effectively hydrolyzed. These results again indicated that the AAPF peptide would also represent an effective irreversible inhibitor when coupled to a chloromethylketone moiety, and we therefore performed studies to determine whether AAPFcmk inhibitor affected growth of C3H/10T1/2 cells. Initial experiments showed that relatively high concentrations of AAPFcmk inhibitor produced substantial inhibition of cell growth; $2.5 \times 10^{-3}$ M and $5 \times 10^{-3}$ M concentrations produced 34% and 56% inhibition of cell growth, respectively. Lower concentrations of AAPFcmk (up to $10^{-5}$ M) had no effect on cellular growth. Exposure of C3H/10T1/2 cells to selected concentrations of AAPFcmk produced inhibitions in NS protease which were similar to those observed with rat liver NS, suggesting a similar $K_i$ (a formal $K_i$ determination was not undertaken), and previous results also indicated that AAPFcmk

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3 The abbreviations used are: NS, nuclear scaffold; MPC, multicatalytic protease complex; ER, endoplasmic reticulum; MCA, 3-methylcholanthrene; DMISO, dimethyl sulfoxide; sb, S-benzyl.
effectively entered the cells and produced a selective inhibition of the NS protease activity (1).

To determine whether the growth inhibition was manifested in a particular stage of the cell cycle, C3H/10T<sub>1/2</sub> cells were harvested after 24, 48, and 72 h of exposure to 0, 25, or 50 μM AAPFcmk. Nuclei were prepared, stained with propidium iodide, and examined for DNA content using flow cytometry (Fig. 1). There was little or no change in distribution of DNA content of the growth-inhibited cells as compared with control cells at any of the times or concentrations tested. These data suggest that AAPFcmk inhibits cell growth throughout the various stages of the cell cycle and thus appear to differ from results with rat hepatoma cells, in which inhibitors of chymotrypsin-like proteases blocked insulin-induced mitotic events in G<sub>1</sub> (26).

Experiments were next undertaken to assess the effect of AAPFcmk on transformation of C3H/10T<sub>1/2</sub> cells by MCA using a standard in vitro assay (27, 28) which has been used previously to demonstrate inhibition of morphological transformation by protease inhibitors from soybean or potatoes (29, 30). In a number of independent experiments, we observed 32 type II/III foci/60 dishes after treatment with MCA (0 were observed in the background control dishes). Inclusion of AAPFcmk in the medium after MCA exposure had profound effects on development of type II/III foci, resulting in 80–90% inhibition of focus formation, even at the lowest concentration tested (Table 1).

Since this assay (without protease inhibitor) yielded less than a single focus per dish (similar to results of others; see Refs. 27–30), we also tested AAPFcmk under more rigorous conditions (31), utilizing a modified, more sensitive assay (i.e., treatment with MCA beginning 5 days after seeding to maximize target cell population) which produces 2–4 foci/dish. Even under these conditions, we observed a marked, dose-dependent quantitative inhibition of type II/III focus formation (Table 1), even with the lowest concentration of AAPFcmk tested (100 μM). Inclusion of irrelevant chloromethylketone inhibitors (Lcmk or FGALcmk, which are based upon pep-
The results of these experiments allowed us to differentiate between types of inhibitors (Table 1). Tides not hydrolyzed by the NS protease had no effect on focus formation (Table 1).

Subsequent experiments were also performed to titrate out the inhibitory effects of AAPFcmk at lower concentrations (Table 2). Inhibition of focus formation was again considerable (and quite similar) at $10^{-10}$ and $10^{-12}$ M concentrations but was markedly reduced when assessed at $10^{-14}$ M, where an inhibition of 13% was observed in the first experiment versus 17% in the second experiment (Table 2; lower concentrations were not tested). Comparison of these results with those from the standard assay at the same concentration (Table 1) reflects the greater sensitivity of detection with the enhanced assay, which is predicated upon the increased number of foci/dish.

In addition, there were obvious qualitative differences in the type II/III foci which developed in the presence of AAPFcmk: resultant foci were generally smaller and less hyperchromatic, and they showed a greater degree of differentiation/morphological organization than typical type II/III foci that developed in the absence of the protease inhibitor (Fig. 2). In fact, in many cases, the foci scored as "type II/III" which developed with AAPFcmk showed largely type I morphology, with small areas demonstrating type II/III morphology (Fig. 2). This result suggests that cellular transformation may have represented a subsequent event which occurred in occasional cells within type I foci in the presence of the protease inhibitor.

Interestingly, at relatively high ($10^{-10}$ to $10^{-8}$ M) concentrations, we also occasionally observed formation of phenotypically abnormal foci, which are scored as type I foci in standard morphological assessment. Upon ring subcloning of these abnormal foci in the absence of AAPFcmk inhibitor, cells exhibited abnormal phenotypic patterns, which consisted predominantly of interlacing bundles of cells in regular arrays, but they did not form type II/III foci indicative of transformed cells. Further characterizations are in progress, but these results suggest that these altered cells may be arrested in an early stage of transformation (32). A similar morphology has been observed with C3H/10T1/2 cells transfected with an activated Ha-ras oncogene (33), in which a second, undefined spontaneous event must occur before progression to a fully transformed state.

We suggest that the NS protease provides a reasonable target to explain the ability of selected protease inhibitors

![Fig. 2. Qualitative changes in foci which develop in the presence of AAPFcmk. Cells were treated with 3-methylcholanthrene and subsequently grown in the absence or presence of AAPFcmk at $10^{-10}$ M. Left, typical background of nontransformed fibroblasts. Center, typical type II/III focus which developed in the absence of AAPFcmk. Right, representative aberrant focus which developed in the presence of AAPFcmk and was scored as type II/III. Most of the focus shows type I morphology, whereas a small peripheral area shows type II/III morphology.](image-url)
to block cellular transformation and carcinogenesis (34–37), including effects of soybean or isolated soybean protease inhibitors on carcinogenesis in rats or mice (35, 38, 39), or inhibition of in vitro cell transformation by the Bowman-Birk protease inhibitor (29, 30, 40). In fact, it has been argued that the critical anticanerogenic feature of the Bowman-Birk inhibitor is its ability to inhibit chymotryptic-like activity (40), so that this inhibitor (or a fragment) may also work upon the NS protease. Perhaps more pertinent, we also point out that although the NS protease shows a substrate preference which is basically similar to chymotrypsin, it also hydrolyzes some trypsin-like substrates quite effectively (i.e., VLKsb and Rsb), as is characteristic of MPC complexes. In fact, this may underlie the reported ability of trypsin inhibitors or antipain (35, 37) to also block transformation or skin carcinogenesis, albeit at somewhat higher concentrations.

Inhibition of ER activity could relate to reports (41, 42) on a Ca\(^{2+}\)-regulated serine protease activity correlatively related to carcinogenesis in rat liver. This protease activity was associated with the ER (nuclear or NS fractions were not examined), showed a chymotrypsin-like substrate preference, and was induced by tumor promoters (42). Furthermore, it was markedly elevated in hyperplastic nodules and hepatocellular carcinomas as compared with surrounding liver (41). However, we consider these ER effects to be less relevant here. First, the effects of AAPFcmk (at 1 \(\mu\)M) observed on the ER activity in fibroblasts occurred later and were significantly smaller than the effects on the NS protease (1). In addition, no inhibition of ER activity has been observed in hepatocyte-derived cell lines (data not shown), and in these cells the effects of AAPFcmk were selective for the transformed phenotype, producing marked growth inhibition of transformed hepatocyte-derived cell lines (expressing an activated human c-Ha-ras) while having no effect on the SV40-immortalized parental cell line.\(^4\) Second, whereas AAPFcmk shows a \(K_i = 56\) nM for NS protease, the \(K_i\) for ER activity is 23 times higher (1.4 \(\mu\)M), making it unlikely that the effects observed with very low AAPFcmk concentrations involve the ER activity.

The NS protease activity may be important for a variety of reasons. First, its ability to preferentially cleave lamins A/C, as well as its Ca\(^{2+}\) regulation, may have importance in the nuclear alterations pertinent to the early stages of carcinogenesis (see also Ref. 43), a contention supported by the dramatic effects of AAPFcmk on fibroblast transformation described here. Second, the chromosomal abnormalities associated with various carcinogens may conceivably involve widespread proteolytic degradation of chromosomal proteins (44, 45). Third, it may somehow be involved in NS breakdown, possibly in normal mitosis, but especially in abnormal/pathological settings such as the early karyolysis observed in acute irreversable cell injury, where lamin phosphorylation is highly unlikely. Fourth, it is ideally suited for degradation of various nuclear oncogenes proteins, many of which (including c-myc, c-fos, p53, and cyclins) show short intranuclear half-lives (46, 47) and which often appear to be degraded by ubiquitin-dependent protease activity, which is another often-described characteristic of MPC complexes (48, 49), depending upon isolation conditions. In addition, many other potentially important proteins (for instance, transcription factors) would also seem to require rapid intranuclear proteolysis.

Materials and Methods

C3H/10T\(_{12}\) mouse embryo fibroblasts were used for these experiments. To examine potential effects on growth rate (or cytotoxicity), C3H10T\(_{12}\) cells were seeded at 2 \(\times 10^4\) cells/plate, and culture medium (basal Eagle’s medium with 10% fetal calf serum, containing various concentrations of AAPFcmk added from concentrated stocks in DMSO) was removed and replaced every other day; cells were then trypsinized and counted after various intervals.

For preparation of NS, C3H10T\(_{12}\) fibroblasts were harvested using a rubber policeman. Cells were homogenized in Teflon-glass homogenizers in 0.25 M STKM2 buffer (sucrose of molarity as specified-50 mM Tris-HCl, pH 7.6-25 mM KCl-5 mM MgCl\(_2\)-5 mM 2-mercaptoethanol) also containing 0.5% Nonident P-40 and 0.5% Triton X-100 detergents. Nuclei were purified through sucrose buffer cushions, aliquots were taken, and NS was then isolated as described (1), with protease inhibitors (20 \(\mu\)g/ml leupeptin-25 \(\mu\)g/ml aprotinin-10 \(\mu\)g/ml pepstatin-0.5 mM benzamidine) included in the sucrose cushion and at all stages of preparation. The DNase digestion step was modified as necessary, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein yields.

For comparative purposes, ER and NS fractions were also isolated from rat liver hepatocytes as described (1), and the \(K_i\) value reported herein was determined as described for NS, with recentrifugation of ER preparations as per initial isolation conditions (1). Protein was quantitated by the method of Lowry et al. (50).

Protease activity was measured in assays with S-benzyl substrates (1). S-benzyl substrates were tested at 0.25 mM final concentrations; standard assays were at 37°C for 2–5 h with 25 \(\mu\)g nuclear, ER, or NS protein in TKMC buffer (50 mM Tris-HCl, pH 7.5-25 mM KCl-5 mM MgCl\(_2\)-10 \(\mu\)M CaCl\(_2\)) as described. Dithiopyridine (at 1 mM concentration) and substrates were added from concentrated stock solutions in DMSO, and appropriate blanks were run concurrently. After incubations, supernatants were filtered and read at 324 nm as described, with an e-value of 19,800/cm-M used for the thyopidine product. Substrate Pxx amino acids (numbered sequentially from right to left) were unmodified (FGALsb, VLKsb, Lsb) or contained methoxysuccinyl (mAAPFsb) or benzoyloxycarbonyl (zYsb, zRZsb, zRsb) modifications.

Transformation assays were conducted with C3H/10T\(_{12}\) cells. Initial experiments utilized a standard assay (27, 28), in which cells were treated with MCA (1 \(\mu\)g/ml) 24 h after seeding. Plating efficiency ranged from 21 to 31%. Treatment with 1 \(\mu\)g/ml MCA resulted in 95–100% survival. Subsequent experiments utilized a modified, more sensitive assay (31). Briefly, 2000 cells/plate were seeded and exposed to the carcinogen MCA at 1 \(\mu\)g/ml for 24 h beginning 5 days after seeding. Following carcinogen exposure, medium (basal Eagle’s medium with 10% fetal calf serum) was removed and replaced with fresh medium which included various concentrations of AAPFcmk in DMSO (DMSO and/or the ineffective inhibi-

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\(^4\) G. A. Clawson, L. L. Norbeck, and H. Isom, manuscript in preparation.
itors Lcmk or FGALcwk were added to controls). Type II and type III foci were scored as previously described, with modifications for scoring pleomorphic and border-line foci (51).

References


