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Identification of N- Terminus Peptide of Human Granulocyte/Macrophage Colony Stimulating Factor as the Site of Nucleotide Interaction

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The interaction of nucleotides with recombinant human granulocyte/macrophage colony stimulating factor (rhGM-CSF) has been investigated. Utilizing nucleotide photoaffinity probes [γ^{32}P]-8N_3ATP and [β'^{32}P]-8N_3Ap_4A, an analog of alarmone, the specificity of interaction was demonstrated by saturation of photoinsertion by these analogs and protection of photoinsertion by these analogs in the presence of natural nucleotide. The site of photoinsertion was tentatively determined to be Ser^9. The photolabeled cytokine has lost most of its biological activity in a cellular proliferation assay, indicating a possible physiological role for this interaction.

Granulocyte/macrophage colony stimulating factor (GM-CSF) is a regulatory cytokine important in the proliferative and functional activation of hematopoietic cells (1). Even though the specific cell surface receptors have been identified and their role in signal transduction has been proposed, many events relating to specific gene expression remain unexplained (2). Since most cytokines and peptide hormones are internalized they may be exposed to the intracellular milieu which contains nucleotides. They may be compared to toxins which are internalized and express their catalytic activity through their interaction with nucleotides. Utilizing nucleotide photoaffinity probes we have demonstrated that several of these polypeptides such as IL-2, rmGM-CSF, glucagon, ANF, prolactin and aFGF possess specific high affinity nucleotide binding sites (3-8).

While, murine and human GM-CSF share 54% amino acid sequence homology (9, 10), they are species specific as far as their biological actions are concerned (11). To understand the possible physiological significance of nucleotide interactions with GM-CSF we have investigated the nucleotide binding properties of rhGM-CSF in order to

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determine if the nucleotide binding is conserved across the species barrier. The cytokine was photolabeled with photoaffinity analogs of ATP and Ap_4A and subjected to proteolytic digest. The photolabeled peptide was then isolated by immobilized metal affinity chromatography and rpHPLC (4, 12, 13). The photolabeled cytokine was found to have lost most of its biological activity as determined by a cellular proliferation assay.

Materials and Methods

Materials: rhGM-CSF (*E. Coli* derived) was from Amgen Inc.. Modified trypsin was from Promega. The photoprobes [γ^{32}P]-8N_3ATP and [β^{32}P]-8N_3Ap_4A were kindly supplied by RPI Corp., Mt. Prospect, IL. All other reagents were from Sigma or Aldrich.

Methods: Photoaffinity Labeling: rhGM-CSF (1.0 µg) was incubated at 4°C with various photoprobes alone for 15 sec, or initially with competitors for 1 min followed by probe for 15 sec in 40 µL of 20 mM NaH_2PO_4, pH 4.5 (photolysis buffer), and photolyzed at 4°C for 45 sec with a hand-held UV lamp (254 nm, 5000 µw/cm^2). The reaction was quenched by the addition of protein solubilizing mixture (4) and the samples were separated by 12% SDS-PAGE. The gel was stained by CBB, destained, dried on a slab gel dryer and exposed to X-ray film. The individual cytokine bands were excized and the radioactivity was determined by liquid scintillation counting.

Isolation of Photolabeled Peptide by Metal Chelate Chromatography: rhGM-CSF (100 µg) was photolabeled twice with probe (probe:cytokine molar ratio of 5:1 in each photolysis) as described above. The photolabeled cytokine was precipitated by adding equal volume of ice-cold perchloric acid and digested with modified trypsin (10% w/w) or Staph V8 (10% w/w) as previously described (4). The immobilized metal affinity chromatography was performed as described elsewhere (4, 12, 13). The photolabeled peptides were eluted with 10 mM phosphate wash.

Reverse Phase HPLC: The photolabeled peptides isolated by immobilized metal affinity chromatography were purified further by rpHPLC as described elsewhere (4). The peak containing radioactivity and indicating corresponding UV absorbance at 214 nm were subjected to amino acid sequence analysis on Applied Biosystems Liquid Phase Protein Sequenator at University of Kentucky Sequencing Facility.

Bioassay: The 8-N_3Ap_4A photolabeled rhGM-CSF samples were prepared by incubating 500 ng of cytokine with 16.5 µM probe for 15 sec at 4°C and then photolyzing for 45 sec at 4°C. This was followed by two more additions of 12.5 and 10 µM probe and photolysis. The control sample was photolyzed for 2.25 min before the probe was added in three additions of 16.5, 12.5 and 10 µM respectively.

MO7e cells were seeded (1X10^4/well) into 96 well microtiter plates which contained rhGM-CSF standards or photolabeled samples in 1:2 serial dilutions starting at 200 ng/mL. The assay mixture in a final volume of 200 µL/well was then incubated for 72 hr at 37°C in a 5% CO_2 humidified incubator and pulsed with [^{3}H]thymidine (1µCi/well) for the final 4 hr. The contents of the wells were harvested onto glass-fiber filters and [^{3}H]thymidine incorporated into DNA was determined.

Results and Discussion

Cytokines have multiple effects, i.e. the same cytokine can have different effect on different cell lineages, or different cytokines may exert the same effect on the same cell type (14). Cytokines are believed to exert their effects through specific receptor

interactions (15). Although some components in the post-receptor signal transduction process have been identified, the complete process leading to specific gene transcription is not clear (2). Most of these cytokines are internalized, which should expose them to the intracellular pool of nucleotides. These peptides also are of appreciable size such that they may possess some kind of enzymatic activity. Nucleotides regulate biochemical processes through their interactions with regulatory proteins. Investigation of nucleotide binding properties of these peptides was based on the hypothesis that they may work like toxins. Toxins are known to be internalized and express catalytic activity involving nucleotides.

Utilizing nucleotide photoaffinity analogs, cytokines such as IL-2, GM-CSF, and peptide hormones such as glucagon, ANF, prolactin and aFGF have been shown to possess specific high affinity nucleotide binding sites (3-8). Most of these lose their biological activity upon photolysis suggesting a possible physiological role for these interactions.

Human and murine GM-CSF share 54% amino acid sequence homology. However, their respective biological activities are highly species specific (9-11). We have already reported that rmGM-CSF specifically binds ATP and Ap_4A, a putative alarmone (4). We have now investigated and report the similar nucleotide binding properties of human GM-CSF.

In various photolabeling experiments GM-CSF from both species showed similar properties. Recombinant human GM-CSF could be photolabeled with photoaffinity analogs of ATP, Ap_4A and GTP. The photolabeling was optimal in 20 mM NaH_2PO_4,

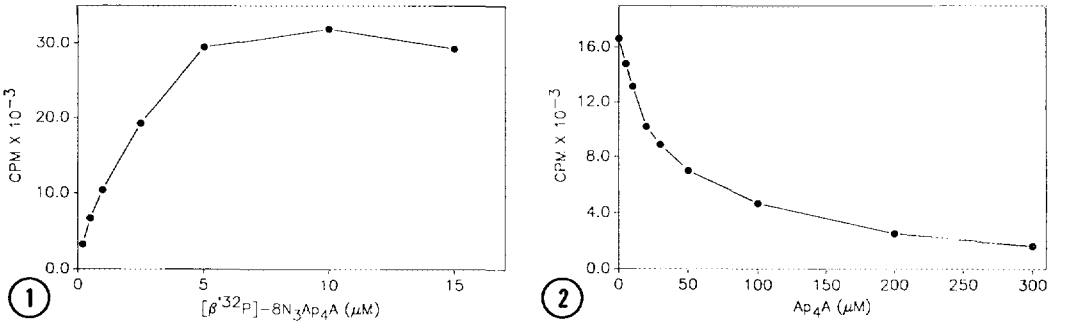

Figure 1. Saturation of photoinsertion of [β'^{32}P]-8N_3Ap_4A in rhGM-CSF: rhGM-CSF (1 μg) in photolysis buffer was incubated with indicated concentrations of [β'^{32}P]-8N_3Ap_4A at 4°C for 15 sec and photolyzed for 45 sec. The samples were analyzed by SDS-PAGE. The stained protein bands excized and radioactivity determined by liquid scintillation counting.

Figure 2. Protection of photoinsertion of [β'^{32}P]-8N_3Ap_4A in rhGM-CSF: rhGM-CSF in photolysis buffer was initially incubated with indicated concentrations of Ap4A at 4°C followed by 20 μM probe for 15 sec and photolyzed for 45 sec. The samples were analyzed as in figure 1.

pH 4.5. Most of the metal ions showed inhibitory effect except $CaCl_2$ and $ZnCl_2$, where a 10-15% increase in incorporation was observed (data not shown). The incorporation decreased in the presence of EGTA (data not shown).

The specificity of nucleotide interaction can be demonstrated by saturation of photoinsertion and protection of photoinsertion by azido analogs in the presence of natural nucleotide at physiologically relevant concentrations. The photolabeling with [β^{32}P]-8N_3Ap_4A saturated at 5-7 μM with an apparent K_d of 2-2.5 μM (Fig. 1). In protection experiment 85% of photoinsertion with 2 μM probe could be protected by 200 μM Ap_4A (Fig. 2). The photoinsertion with [γ^{32}P]-8N_3ATP saturated at 50-55 μM with an apparent K_d of 10-12 μM (data not shown). Approximately 75% of photoinsertion with 20 μM probe could be protected in the presence of 600 μM ATP (data not shown). These results demonstrated that rhGM-CSF showed affinity for ATP and Ap_4A similar to rmGM-CSF (4). In various experiments 20-40% of the cytokine was modified as

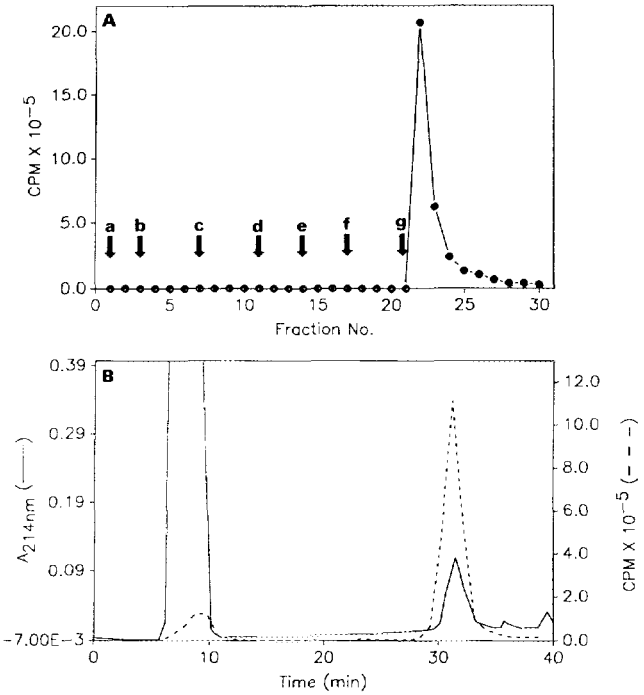

Figure 3. Isolation of photolabeled peptide: rhGM-CSF (100 μg) was photolabeled with probe and digested with V8 protease as described in the methods section. **A.** The immobilized Fe^{+3} affinity resin (600μL) was prepared as described elsewhere (12). The digest was a, loaded onto the column; washed with b, 100 mM ammonium acetate, pH 8.0 (buffer A); c, with buffer A containing 0.5 M NaCl; d, buffer A; e, buffer A containing 2 M urea; f, buffer A; g, buffer containing 10 mM K_2HPO_4. **B.** Fractions 22-27 from A containing photolabeled peptides were pooled, concentrated and purified by rpHPLC as described earlier (4). Fraction 32 was submitted for amino acid sequence analysis.

assessed by SDS-PAGE. This is a minimum number since label incorporated is relatively unstable to gel electrophoresis procedures.

The photolabeled peptide was isolated and identified by a combination of immobilized metal (Fe^{+3} or Al^{+3}) affinity chromatography and rpHPLC procedures (4, 12, 13). Fig 3A shows radioactive elution profile for rhGM-CSF photolabeled with [β'^{32}P]-$8N_3Ap_4A$, digested with V8 protease and purified by immobilized Fe^{+3} affinity chromatography. Most of the radioactivity was retained on the resin which could be eluted with phosphate wash. Fig 3B shows radioactivity and 214 nm elution profile for reverse phase HPLC purification of fractions 22-27 from fig. 3A. A single radioactive peptide was isolated in this and all other peptide isolation experiments. The sequence analysis from various experiments is summarized in Table 1. Based on the pmol recovery the site of modification is tentatively assigned to be on Ser^9. In the case of murine GM-CSF a peptide in the same region was identified indicating conserved binding motif in both species (4).

The photolabeled cytokine had lost most of its bioactivity as measured by cellular proliferation assay using the hGM-CSF dependent MO7e cell line (16) (Fig. 4 for $8N_3Ap_4A$ labeled cytokine). The photolabeled and non-photolabeled samples showed a difference in unit activity of approximately 1.5 dilutions. For the samples photolabeled with $8N_3ATP$ the difference was approximately 3 dilutions (data not shown). This indicates a potential physiological significance of these interactions. In the case of

Table 1: **Amino acid sequence analysis of photolabeled peptides**

Cycle No.	Amino Acid (pmol)		
	8-N_3Ap_4A		8-N_3ATP
	Trypsin[c]	V8[c]	V8[d]
1	^{5}S(249)	^{1}A(372)	^{1}A(525)
2	P(401)	P(246)	P(293)
3	S(139)	A(211)	A(331)
4	P(282)	R(a)	R(109)
5	S(97)	S(102)	S(99)
6	T(202)	P(215)	P(211)
7	Q(156)	S(102)	S(99)
8	P(69)	P(134)	P(152)
9	W(b)	S(a)	S(86)
10	E(37)	T(93)	T(65)
11	H(12)	Q(69)	Q(72)
12	V(37)	P(22)	P(25)
13	N(26)	W(b)	W(b)
14	^{18}A(28)	^{14}E(6)	^{14}E(12)

[a] could not be detected.
[b] pmol could not be determined.
[c] purification by immobilized Fe^{+3} affinity chromatography.
[d] purification by immobilized Al^{+3} affinity chromatography.

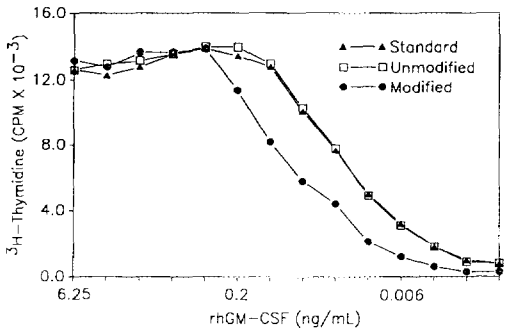

Figure 4. Bioassay of rhGM-CSF photolabeled with $8N_3Ap_4A$: The photolysis and bioassay procedure is as described in the methods; (▲) standard rhGM-CSF, (□) rhGM-CSF photolyzed in the absence of photoprobe and then probe added to it, (●) rhGM-CSF photolabeled with the photoprobe.

rmGM-CSF we have observed the loss of bioactivity due to inability of photolabeled cytokine to bind to the receptors[1]. Such a possibility exists for rhGM-CSF and is being investigated. Our results indicate that the role played by the nucleotide interaction may be in the dissociation of cytokine bound to the receptor once it is internalized. We have also observed an apparent enzymatic activity for GM-CSF which results in the ^{32}P incorporation inot the cytokine when it is incubated with [α or γ^{32}P]ATP under physiological conditions (17).

The results of this study show similar nucleotide binding properties for both species indicating that the region involved is probably not contributing to the species specificity. However, conservation of nucleotide binding across the species barrier supports a possible physiological role for these interactions.

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