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# NEUTROPHIL ACTIVATING PEPTIDE-2 BINDS WITH TWO AFFINITIES TO RECEPTOR(S) ON HUMAN NEUTROPHILS

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SUMMARY: Neutrophil receptor(s) for neutrophil activating peptides 1 and 2 were studied by competition binding experiments with radiolabeled NAP-1 and NAP-2 preparations. NAP-1 bound with one affinity, NAP-2 with two quite different affinities, to common receptor(s) on neutrophils. Concentrations of NAP-2 needed to induce exocytosis of ß-glucosaminidase corresponded to the higher dissociation constant of the two binding equilibria. Thus, the binding of NAP-2 to PMN with high affinity does not activate the cells. 

NAP-1 and NAP-2 are members of a growing family of small proteins which are thought to play important roles in acute and chronic inflammation (1). NAP-1 and NAP-2 in particular are potent chemoattractants and activators of granulocytes (1). NAP-1 is produced by several different tissues as a mixture of two polypeptides, one consisting of 72, the other a N-terminal extended form of 77 amino acids (2). NAP-2 is a truncated form of platelet connective tissue activating peptide III, which is derived by cleavage of the first 15 N-terminal residues (3). NAP-1(72aa), NAP-1(77aa) and NAP-2 interact with the same receptor(s) on PMN (1,2,4,5). Our objective for this study was to characterize NAP-1 and NAP-2 receptor(s) on PMN by competition binding experiments using radiolabeled preparations of both ligands.

## MATERIALS AND METHODS

Labelling of NAP-1 and NAP-2: Recombinant human NAP-1 and NAP-2 were labeled with $^{125}I$ by the IODO-GEN procedure (Pearce),

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Abbreviations: PMN, polymorphonuclear leukocytes; aa, amino acids.

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resulting in specific activities of 200-220 Ci/mmole for NAP-1 and 70-100 Ci/mmole for NAP-2 as measured by self displacement analysis (6). NAP-2 does not contain aromatic aminoacids and is most probably iodinated at its two histidines (7).

Human PMN preparation: PMN were prepared by lymphoprep gradient centrifugation followed by dextran sedimentation.

Binding assays: Cells were incubated with NAP-1 or NAP-2 in Eppendorf tubes at 0 - 4° for two hours or at 37°C for one hour. Cells were separated from unbound radioactivity by centrifugation through 200 µl fetal calf serum in 400 µl Beckman tubes. Tubes were frozen at -70° and cell sediments were sliced off with scissors and counted in a γ-counter.

NAP-1 and NAP-2 assays: $2\text{-}4 \times 10^6$ PMN in 200 µl colourless RPMI medium containing 2,5 mg/ml bovine serum albumin were pretreated for 5 min at 37°C with 5 µg/ml cytochalasin B and then incubated for 30 sec with NAP-1 or NAP-2 at 37°C. Cells were sedimented for 10 sec in an Eppendorf centrifuge. 100 µl of supernatants were mixed with 100 µl of 0.2 mM substrate 4-methylumbelliferyl N-acetyl-ß-D-glucosaminide (Sigma), and the developing fluorescence was measured with a Titertek Fluoroskan II, Flow Laboratories.

## RESULTS

NAP-1 and NAP-2 are not degraded when bound to PMN: Iodinated preparations of both chemotactic factors electrophoresed as single bands before and after they had bound to cells (Fig. 1). This result shows that the ligands were not degraded or processed when bound to PMN at 0 - 4°C.

NAP-1 and NAP-2 have different specific biological activities: Specific biological activities of the two NAPs differed considerably when measured by their potency to release ß-glucosaminidase from PMN. NAP-1 was 100 fold more active than NAP-2 (Fig. 2).

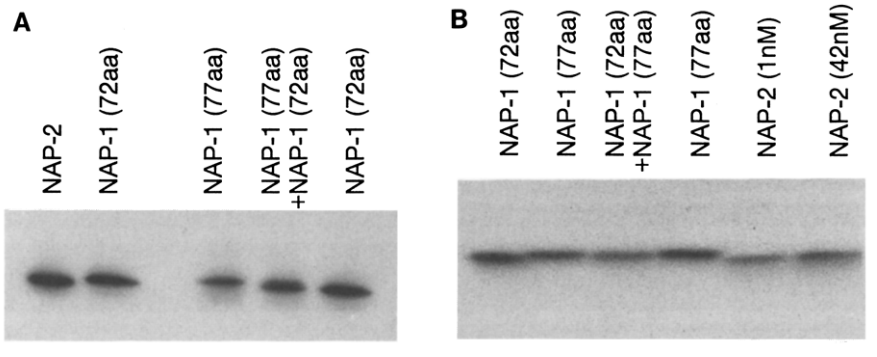


Fig. 1. SDS-polyacrylamide gel electrophoresis and autoradiography of iodinated recombinant human NAP-1 and NAP-2. A) Before binding to cells, B) cell-bound iodinated NAP preparations after elution from the cells by pH 3 treatment. [$^{125}$I]NAP-2 was eluted from cells that had been incubated with low (∿1nmole/L) and high (∿42nmole/L) concentrations of the radioligand, respectively.

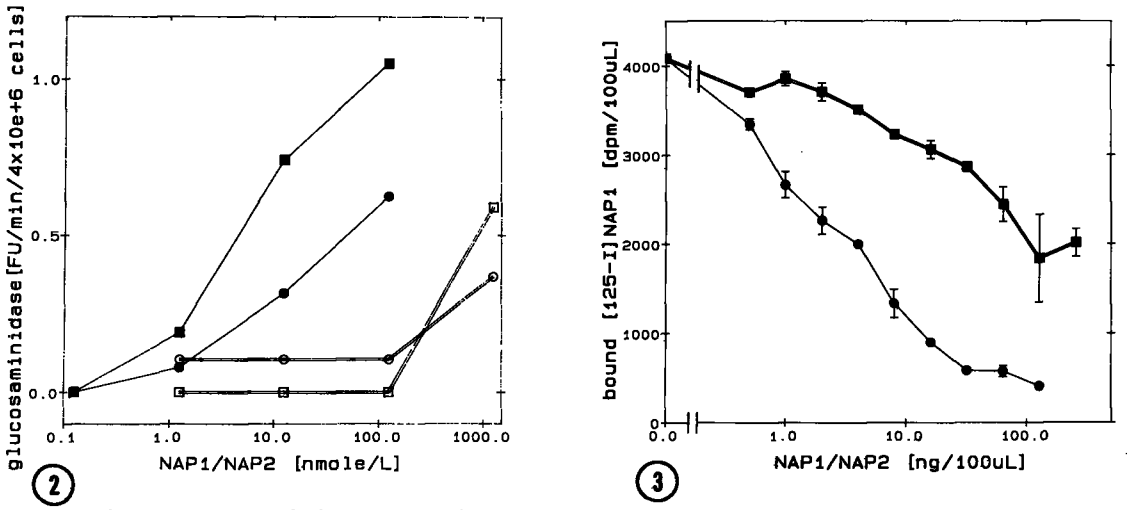


Fig. 2. Specific biological activities of NAP-1 and NAP-2. ß-glucosaminidase release from stimulated PMN was measured in dependence of increasing NAP-1 ——— , and NAP-2 ═══ concentrations. Data from experiments with PMN of two donors are presented, ● , o donor 1, ■ , □ donor 2. FU = fluorescence units; a blank value of 0.17 FU/min for the medium control is substracted.

Fig. 3. Competitive inhibition of binding of [$^{125}$I]NAP-1 by unlabeled NAP-1 ● and NAP-2 ■ . The result of one single experiment, performed in duplicate at 0 - 4°C is given. The concentration of [$^{125}$I]NAP-1 was 0.5 nmole/L.

NAP-1 and NAP-2 bind to the same receptor(s) on PMN; complex binding pattern for NAP-2: NAP-1 and NAP-2 competed for the same receptor(s) on PMN as shown by the nearly complete inhibition of binding of [$^{125}$I]NAP-1 by unlabeled NAP-2 and of [$^{125}$I] NAP-2 by unlabeled NAP-1 (Fig. 3-6). Experiments performed at 0.4°C and 37°C lead to comparable results. Quantitatively, results from experiments with cells of different donors varied somewhat. Accordingly, the standard deviations are high. To achieve 50% inhibition of [$^{125}$I]NAP-1 binding roughly 50 - 100 times more NAP-2 had to be given when compared to NAP-1 (Table). The displacement of [$^{125}$I]NAP-1 by NAP-2 lead to a biphasic and shallow curve which reflects complex ligand-receptor interactions (Fig. 3,4). This became even more obvious when the reciprocal experiment was performed, e.g. displacement of [$^{125}$I]NAP-2 by unlabeled NAP-2 or NAP-1 (Fig. 5,6). At low concentrations of the radioligand (0.8 - 1.6nmole/L), NAP-1 and NAP-2 competed with similar high affinity for binding sites. In fact, in repeated experiments, NAP-2 was more effective than NAP-1 (Fig. 5, Table). However, at high concentrations of [$^{125}$I]NAP-2 (42nmole/L),

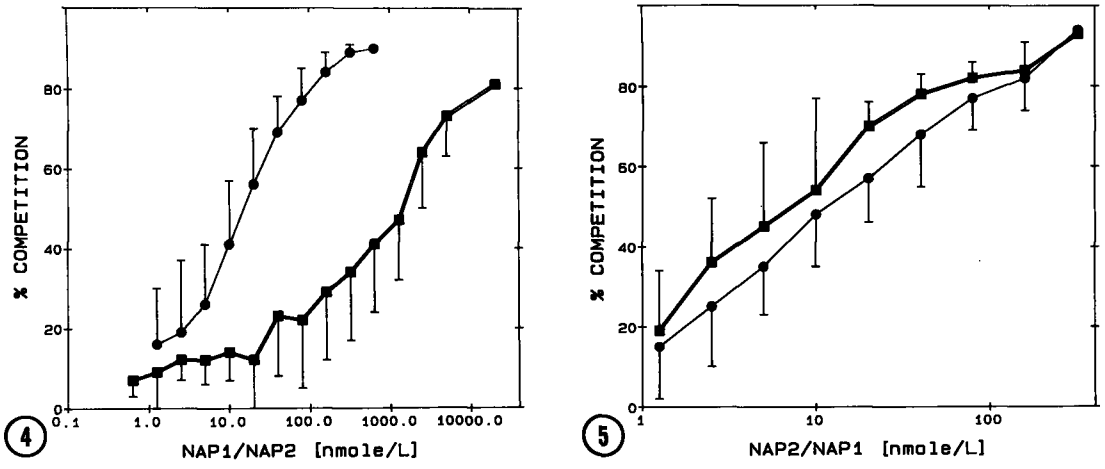


Fig. 4. Competitive inhibition of binding of [$^{125}$I]NAP-1 by unlabeled NAP-1 ● and NAP-2 ■ . Data of experiments at 0 - 4°C (n = 6) and 37°C (n = 4) were taken together. Concentrations of [$^{125}$I]NAP-1 varied between 0.5 - 0.9 nmole/L. Bars indicate standard deviation.

Fig. 5. Competitive inhibition of binding of [$^{125}$I]NAP-2 by unlabeled NAP-2 ■ , and NAP-1 ● . Data of six independent experiments, four at 0 - 4°C, two at 37°C, are summarized. Concentrations of [$^{125}$I]NAP-2 varied between 0.8 and 1.6 nmole/L. Bars indicate standard deviation.

NAP-1 was a more potent competitor than NAP-2 (Fig. 6). Complete competition in the latter experiments was not reached, since at these high concentrations of [$^{125}$I]NAP-2, unspecific binding

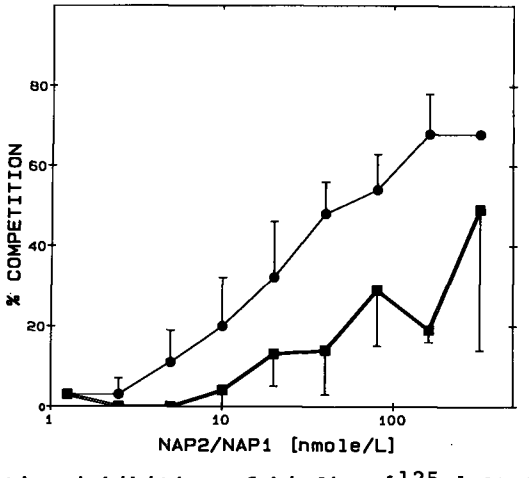


Fig. 6. Competitive inhibition of binding [$^{125}$I]NAP-2 by unlabeled NAP-2 ■ and NAP-1 ● . Data of two independent experiments, each performed in duplicate at 0 - 4°C, are summarized. Concentration of [$^{125}$I]NAP-2 was 42 nmole/L.

Table 1. $IC_{50}$ values of all competition experiments performed

| Combination | IC 50 (nmol/L) | n |
|---|---|---|
| (125I) NAP-1 (0,7nM) / NAP-1 | 17 ± 10 | (7) |
| (125I) NAP-2 (1,2nM) / NAP-1 | 13 ± 6 | (6) |
| (125I) NAP-2 (1,2nM) / NAP-2 | 7 ± 4 | (5) |
| (125I) NAP-1 (0,7nM) / NAP-2 | 1300 ± 1000 | (10) |
| (125I) NAP-2 (42nM) / NAP-2 | 130 ± 115 | (2) |
| (125I) NAP-2 (42nM) / NAP-1 | 46 ± 14 | (2) |
| (125I) NAP-1(72aa) (0,5nM) / NAP-1(72aa) | 16 ± 10 | (3) |
| (125I) NAP-1(77aa) (0,44nM) / NAP-1(72aa) | 13 ± 4 | (3) |
| (125I) NAP-1(72aa) (0,5nM) / NAP-1(77aa) | 47 ± 23 | (3) |
| (125I) NAP-1(77aa) (0,44nM) / NAP-1(77aa) | 47 ± 24 | (3) |

$IC_{50}$ values were obtained by transformation of inhibition curves as in Figures 3 - 6, into reciprocal Hill plots (8); n = numbers of experiments performed.

was already considerable (see Fig. 7). Our results show that NAP-2 binds with two grossly different affinities to PMN. In saturation binding experiments at equilibrium with [$^{125}$I]NAP-2 using Scatchard transformation of the data, two affinities for NAP-2 binding were also observed (Fig. 7).

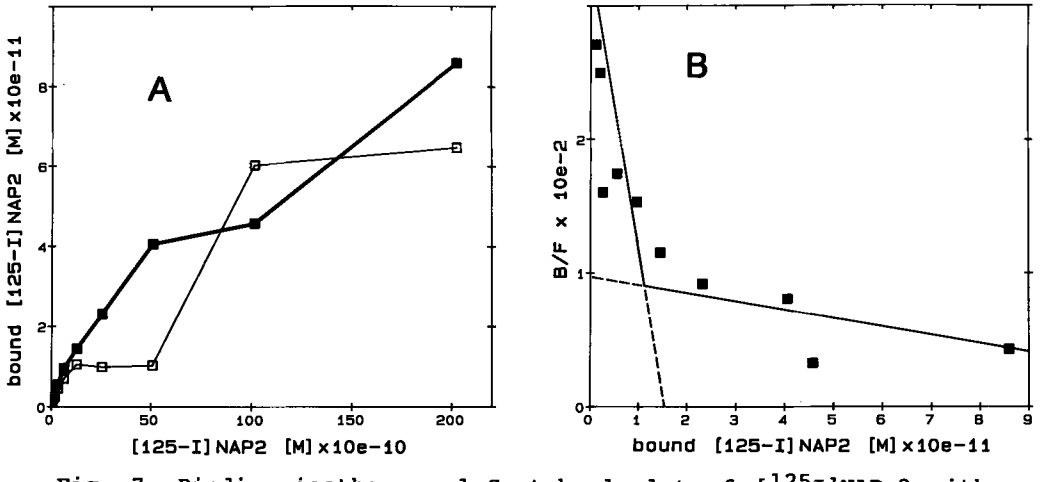


Fig. 7. Binding isotherm and Scatchard plot of [$^{125}$I]NAP-2 with PMN. A: Binding isotherm at 0 - 4°C, B: Scatchard transformation of the data in A. Specific ■ , unspecific □ binding.

## DISCUSSION

Results from our binding experiments with iodinated NAP-1 and NAP-2 preparations and PMN confirm published data that both ligands share common receptor(s) (1,2,4,5). We show in addition that unlabeled NAP-1 fully competes for [$^{125}$I]NAP-2 binding, as unlabeled NAP-2 does for [$^{125}$I]NAP-1. This complete crosscompetition excludes specific receptors for NAP-1 or NAP-2 on PMN. However, NAP-2 apparently binds with two affinities to the receptor(s), whereas NAP-1 binds only with one, the high affinity. This conclusion is based on the findings that 1) NAP-1 displaces both ligands equally well, whereas NAP-2 needs much higher concentrations to displace NAP-1 than NAP-2 (Table), 2) competition for binding of [$^{125}$I]NAP-1 by unlabeled NAP-1 leads to sigmoidal curves, by unlabeled NAP-2 to biphasic and shallow curves [Fig. 4, and (5)], and 3) at high, but not low, concentrations of radiolabeled NAP-2, when it binds with low affinity, NAP-1 is the better competitor. We did not deduce from $IC_{50}$ values dissociation constants and numbers of high and low affinity binding sites. Our binding data are not sufficient to decide whether the receptor(s) or ligands or both exist in bivalent forms. In addition, in a situation of complex interactions of receptors and ligands with more than one affinity, equilibria are both dependent on ligand/receptor concentrations (see Table) and time of incubation (8). However, as a control, we performed competition binding experiments under identical experimental conditions with NAP-1 and the 77aa long precursor form of NAP-1. We obtained sigmoidal and complete reciprocal displacement curves (Table) as was expected for this closely related pair of ligands (2). We conclude that either the receptor(s) for NAP-1/NAP-2, or the ligand NAP-2 exist in bivalent forms and the different equilibria between the different forms lead to the complex binding phenomena which we described.

When specific biological activities and receptor affinities of NAP-2 are compared it becomes obvious that only the low affinity binding of NAP-2 leads to an activation of PMN [compare Fig. 2 with Fig. 4, and (4)]. We have no simple explanation at the moment why high affinity binding of NAP-1, but not of NAP-2, activates the cells. We can, however, conclude that in order to activate PMN by NAP-2 contact of one ligand with one receptor does not seem to be sufficient, a third component, either a second receptor or a second form of the ligand must

cooperate. In addition we predict that NAP-2 behaves as a competitive inhibitor of NAP-1, since it binds with high affinity to PMN without activating the cells. We are testing this possibility at the moment and preliminary results indeed show that NAP-2 interferes negatively with NAP-1 activity as measured by enzyme release by PMN. In vivo, the main function of NAP-2 thus may be not to activate PMN, but to inhibit NAP-1 activity in situations where both mediators are present.
In conclusion, we have shown that NAP-1 and NAP-2 bind to the same receptor(s) on PMN and that NAP-2 binds with high and low affinity.

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